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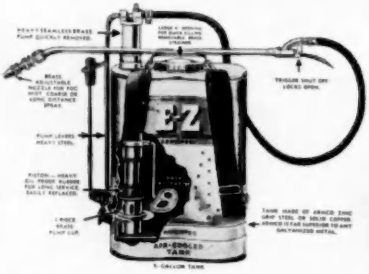

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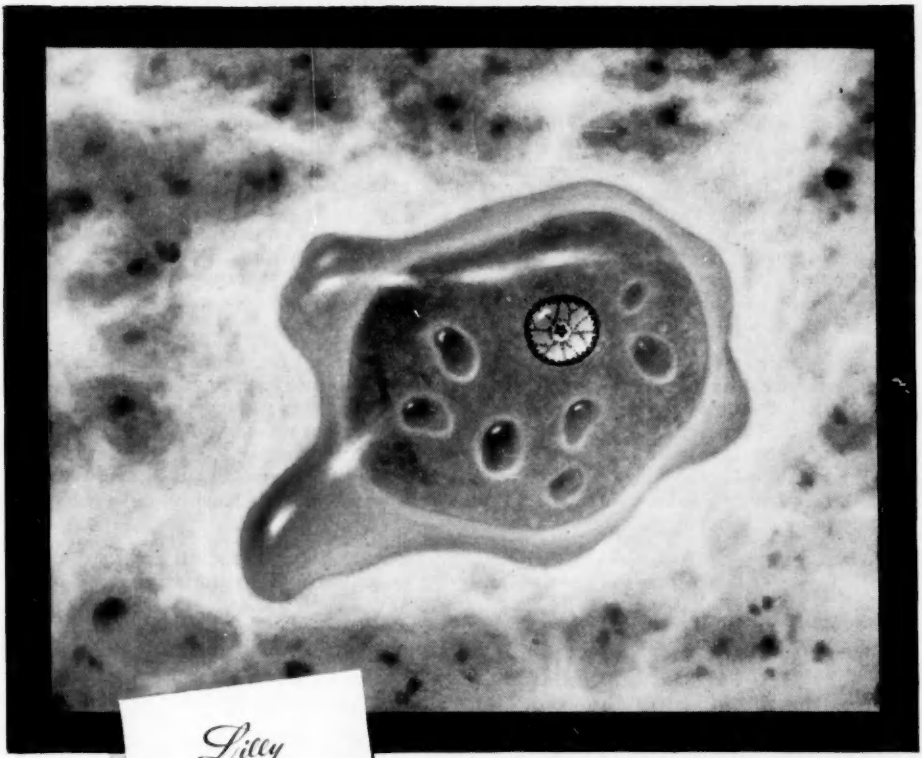

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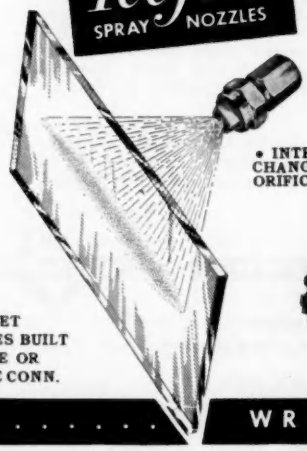

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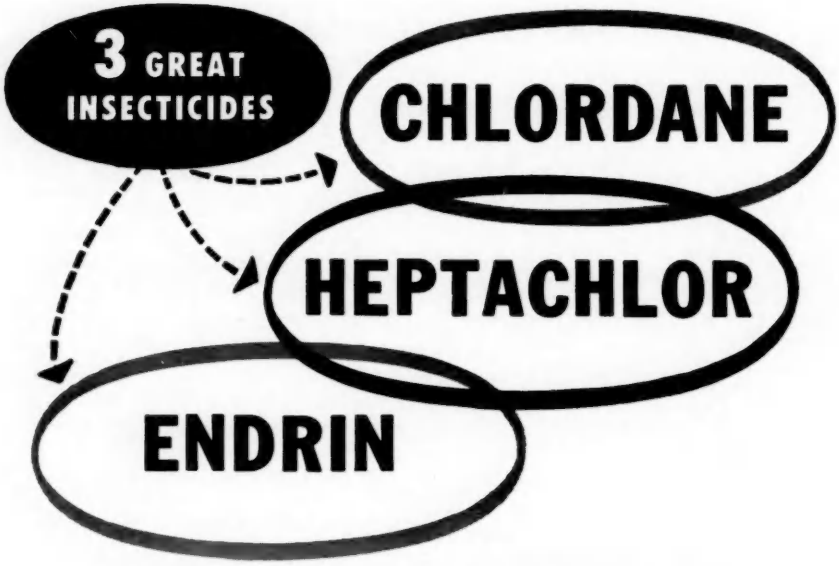

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First of all, I should like to express my feeling of honor and pleasure in serving as President of your Society during the past year. I wish also to express my great appreciation to those who have done the real work: the Council, the various appointed committees of the Society, and especially our Secretary-Treasurer, Dr. Larsh. It has been a pleasure to be associated with each of them.

In selecting the subject for my presidential address, I hope you will not miss the broad philosophical coverage achieved by the past three presidents but will be content with a more restricted research objective. The spleen, lymph nodes and probably all lymphatic tissue possess unique phagocytic, cytopoietic and antibody-forming capacities which are functional in immunity. Today I should like to summarize certain aspects of these functions as they relate to the spleen. Most of my examples will be taken from my own work and that of my coworkers over the past 30 years.

The immunological functions of the spleen are chiefly directed toward invading organisms and other antigens which occur in the blood because its macrophages are oriented to remove materials from the blood. The spleen, therefore, should be compared with other filter organs of the blood, i.e., the liver, bone marrow, adrenal and pituitary. Of these, the liver, probably because of its size, is of greatest importance phagocytically (see later discussion), but, although it synthesizes serum globulins, is probably unimportant in antibody formation (Miller and Bale, 1954; Miller *et al.*, 1954; Thorbecke and Keuning, 1953) except after lymphatic tissue appears in the periportal connective tissue as frequently happens during intense immunization or infection (cf. Thorbecke, 1954, and Coons *et al.*, 1955). The bone marrow forms antibody, but how much is not known. Although relatively insignificant phagocytically, it supplies most of the heterophils which act as a first line mobile defense in certain infections. The heterophil, however, even in pyogenic infections, is probably less important than the macrophage (Taliaferro, 1949b). The adrenal and pituitary are erratic as phagocytic centers and are not important sites of the cytopoiesis of connective tissue cells involved in immunity. To them have been ascribed various functions in the destruction of lymphoid cells and the release of antibody, but the extent and true significance of the pituitary-adrenal-cortical hormones in immunity still needs clarification (Taliaferro, 1949b). Thus, it is evident that the spleen is not unique among the filter organs in any single function concerned in immunity, but is unique in the heterogeneity of its functions. I shall consider in order its phagocytic function, its cytopoietic activity including the production of lymphoid cells and macrophages, its role in the formation of specific antibodies and certain little understood regulatory actions on nonsplenic antibody-forming sites.

PHAGOCYTOSIS

The importance of splenic macrophages in removing particulate and colloidal materials, including invading organisms, is too well-known to need extended discussion. It is illustrated by the studies made on malaria during the years 1884 to 1888 by Laveran, Golgi, Metchnikoff and Marchiafava and Celli (reviewed by Taliaferro and Mulligan, 1937) and by the detailed correlation of phagocytosis with innate and acquired immunity which my colleagues, Cannon, Mulligan, Bloom, Singer, Mrs. Taliaferro and I have reported (see especially Singer, 1954; W. H. and L. G. Taliaferro, 1955; and review by Taliaferro, 1949a). The following facts are worthy of special note. For all practical purposes, phagocytosis of malarial parasites, host cells and cellular debris is limited to macrophages of the spleen, liver and bone marrow. Those of the spleen are individually most active, but the liver because of its size is more important. The phagocytic supremacy of the liver is borne out by other studies on such diverse materials as the uptake of leishmania in the hamster (Stauber, 1955), radio-active colloidal gold[191] in rabbits (Barrow *et al.*, 1951) and radioactive S^{35}-sulfanil-azo-sheep-red-cell stromata in rabbits and mice (Ingraham, 1955). After injecting radioactive stromata intravenously into rabbits, Ingraham found that the liver contained 16 to 70 times as much radioactivity as the spleen, but that the concentration in the spleen was 1.4 to 5.6 times that of the liver. Macrophages of the bone marrow are individually and numerically comparatively inactive. Phagocytosis, moreover, has been correlated with the death of the malarial parasites (Fig. 1) during innate immunity and especially during acquired immunity when individual macrophages become enormously more active due chiefly to the acquisition of opsonins and, secondarily, to nonspecific factors (Taliaferro and Cannon, 1936, Coggeshall and Kumm, 1937, and review by Taliaferro, 1948).

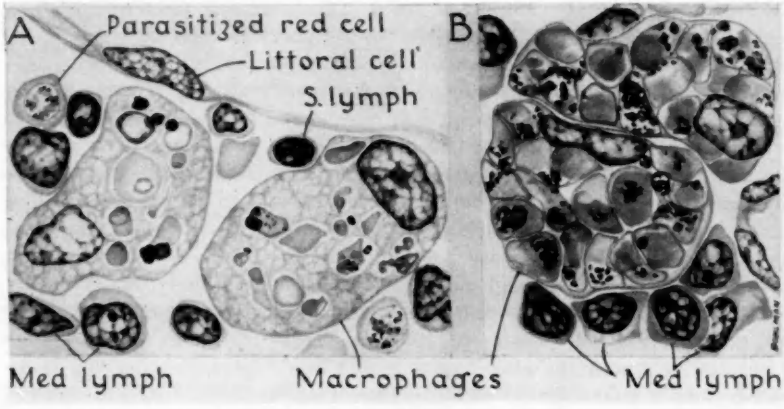

FIG. 1. Comparative degree of phagocytosis of malarial material by splenic macrophages from infections with *P. brasilianum* in cebus monkeys, (A) before and (B) after the supervention of acquired immunity. × 1600. (Redrawn from Taliaferro and Cannon, 1936.)

CYTOPOIESIS AND MESENCHYMAL RESERVES

Reticular cells of the spleen and bone marrow and littoral cells lining the sinusoidal vessels, although immediately available for removing material from the blood, are inadequate except for the mildest infections. Increased numbers of phagocytes, therefore, are of critical importance especially in the nonimmune host early in infections before opsonins increase the effectiveness of individual phagocytes. The production of new phagocytes is one aspect of the opposed processes of cell destruction and cytopoiesis which proceed simultaneously in most infections and, to a less extent, during immunization procedures. It is also a part of the larger question of mesenchymal reserves (Taliaferro 1949b and W. H. and L. G. Taliaferro, 1955).

Mesenchymal reserves in the postnatal animal comprise those cells which retain the mesenchymal capacity to develop into other connective tissue cells. The most important are (1) predominantly fixed cells, such as reticular cells of reticular tissues, littoral cells lining the sinusoidal vessels of the reticular tissues, liver, adrenal and pituitary and adventitial cells or pericytes of loose connective tissue and (2) free cells, chiefly lymphocytes and their lymphoid relatives. These cells are the source of red cells, white cells, both polymorphonuclear and mononuclear phagocytes, and connective tissue cells for mechanical support. In the spleen, they consist of the predominantly fixed reticular cells and the free cells, chiefly lymphocytes, and, at times in many species, various lymphoid stem cells of the myeloid series (Taliaferro, 1949b).

Beginning at an early stage in most infections, there is an interplay between destructive processes, which cause a depletion of macrophages and various lymphoid cells, and reparative processes, which replenish and often during recovery overregenerate to produce hyperplasias of various cellular elements. Thus, in *Plasmodium gallinaceum* in the chicken, the destructive processes, on the one hand, are represented by the following. Macrophages often become so engorged and distended that they eventually disintegrate. As a consequence, the remains of many parasites pass through several macrophages before being finally disposed of. Red cells are destroyed to the extent that profound anemia occurs. Large numbers of lymphocytes succumb to lymphocytocidal factors produced by the infection. Some granulocytes are also injured. Lymphoid cells, furthermore, disappear as a result of their development heteroplastically into macrophages, plasma cells and, in some species, various myeloid cells. The reparative processes, on the other hand, are illustrated by the following. The unengorged reticular cells or fixed mesenchymal reserve proliferate. These cells, however, are inadequate to meet the "demand" for macrophages and are quickly surpassed in importance by the proliferation of the lymphoid cells or mobile mesenchymal reserves (W. H. and L. G. Taliaferro, 1955). The heteroplastic development of lymphocytes into macrophages was first described by Maximow in 1902 (review by Bloom, 1938). The sequence of intense lymphocytopoiesis in the nodules of the spleen during malaria and the migration of lymphocytes into the red pulp where they transform into macrophages was first suggested by Taliaferro and Cannon (1936) and was described in detail by Taliaferro and Mulligan (1937).

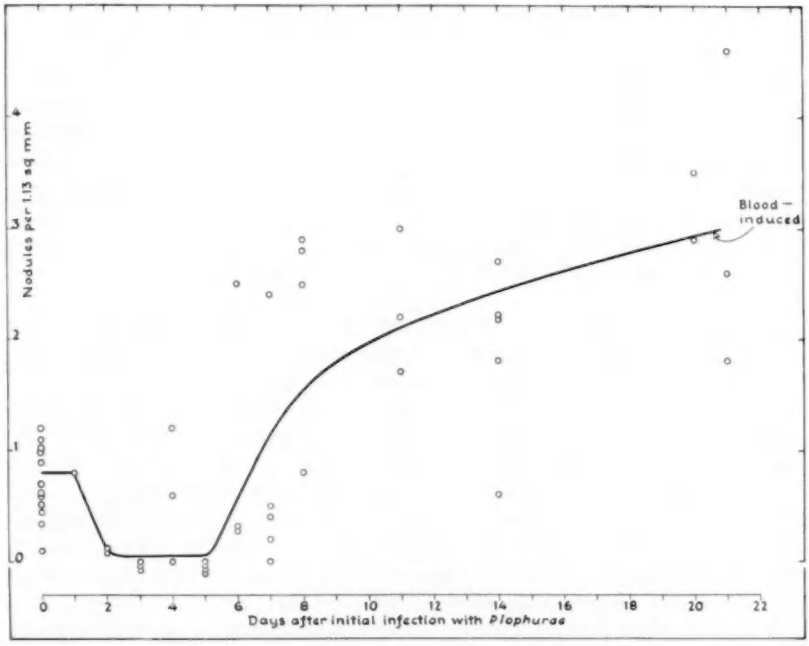

FIG. 2. Changes in the mean number and the range in individual chickens of lymphatic nodules in the spleen during 21 days of an initial blood-induced infection of chickens with *P. lophurae*. The number of nodules in normal chickens is shown at day 0. Each data point represents one chicken (From W. H. and L. G. Taliaferro, 1955.)

In addition, the lymphoid cells become stem cells which replace other cells, such as granulocytes and red cells, and form plasma cells. They also, possibly in conjunction with the fixed cells, probably form antibodies.

The interplay of lymphocytopoiesis and lymphocyte depletion was first clearly described bd Conway (1938 and 1939a) in her work on *Listeria monocytogenes*. We (W. H. and L. G. Taliaferro, 1955) have found that it is well-illustrated by the fall and rise of lymphatic nodules in the spleen during the course of chicken malaria. As seen in Figure 2, uninfected chickens possess a mean of 0.8 nodules per 1.13 sq. mm. in 10 μ sections. This figure decreases to 0.2 nodule during the acute rise and crisis of blood-induced infection with *Plasmodium lophurae*. From the 15th to the 30th day, regeneration accounts for an above normal value of 2.9 nodules, and moderate lymphoid hyperplasia occurs between 1 and 2.5 months with a 2.3 times increase over the normal number. Moreover, as shown in Figure 3, the above normal complement of nodules in latently infected animals when superinfected rapidly decreases but maintains a higher than normal baseline. Ten days after the challenge infection, the nodules increase in number but do not surpass the preballenge level.

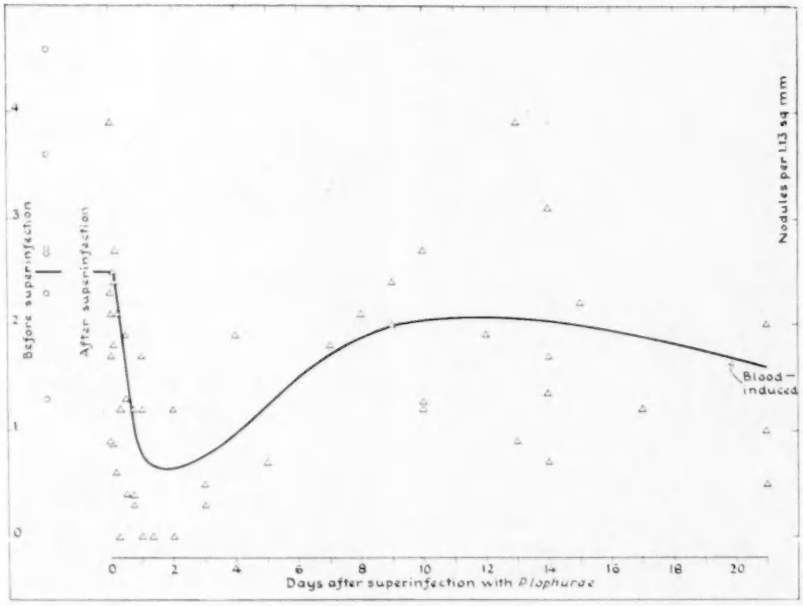

FIG. 3. Changes in the mean number and the range in lymphatic nodules during 21 days following superinfection with *P. lophurae*. (From W. H. and L. G. Taliaferro, 1955.)

Changes in the number of nodules closely parallel general observations on the rise and fall of lymphocytes in the nonnodular dense lymphatic tissue and red pulp of the spleen. In addition, areas previously characterized by a lymphoid hyperplasia often lose their lymphocytes with a consequent exposure of the cellular reticulum (Fig. 4) located around the Schweigger-Seidel sheaths in chickens infected with malaria or at the edge of the white pulp in mice infected with *Trypanosoma cruzi*. Especial attention is drawn to these areas because areas like them are often purported in the literature to represent reticulo-endothelial hyperplasia. Actually, they do not *necessarily* contain an extra number of reticular cells, but the reticular cells are exposed because of the absence of lymphocytes. The fact that lymphoid cells in superinfected chickens do not decrease below the normal level suggests that lymphoid hyperplasia furnishes a cushion and is of direct significance in acquired immunity. Antibodies, however, probably play the dominant role in arresting the superinfection and in preventing a wholesale destruction of lymphocytes. Thus, Conway (1939b) was able to inhibit the lymphocytocidal activity of diphtheria toxin in vivo with diphtheria antitoxin.

Moulder and I (1955) have studied the respiration of spleen slices from chickens infected with *Plasmodium gallinaceum*. We found that there is no detectable change in the nature or rate of carbohydrate metabolism per unit weight of spleen

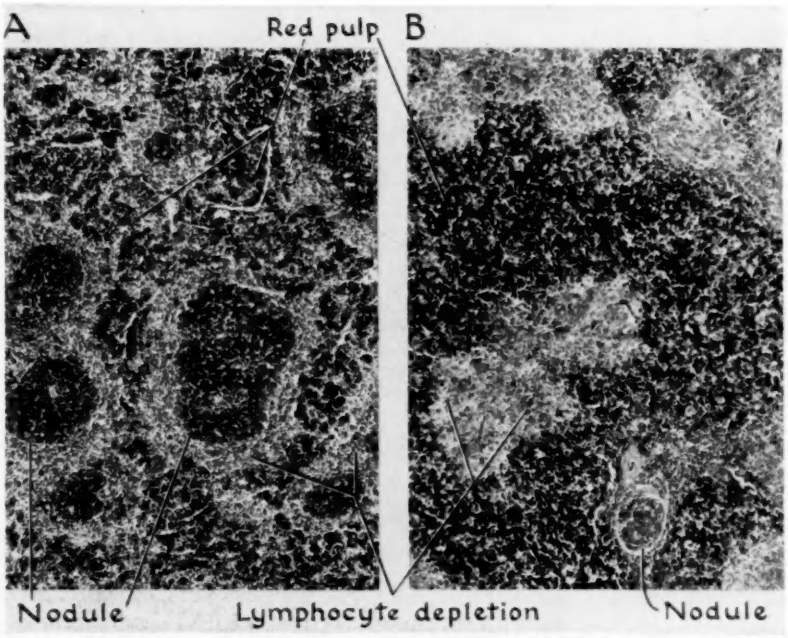

FIG. 4. Marked lymphoid depletion in areas that previously contained dense lymphatic tissue arising as part of the lymphoid hyperplasia of infection. (A) Lymphocyte depletion in the transitional zone of an enlarged lymphatic nodule in an immunized mouse challenged 10 days previously with virulent *Trypanosoma cruzi*. × 40. (B) Lymphocyte depletion in the area surrounding the Schweigger-Seidel sheaths which makes the cellular reticulum clearly visible. From an 8-day blood-induced infection with *P. gallinaceum* × 80. (A, from Taliaferro and Pizzi 1955; B, from W. H. and L. G. Taliaferro, 1955.)

slice during the progressive and regressive histological changes associated with the rise and fall of the infection or the development of acquired immunity. Specifically, there is no change in oxygen uptake, respiratory quotient, anaerobic glycolysis or glucose utilization throughout the infection. These observations, however, do not preclude the possibility of some increase in the metabolic rate of individual living cells at the time of intense immune reactions when many dead cells are seen in the tissue sections. The findings indicate that the hypertrophy of the spleen involves the formation of normally functioning new tissue.

In considering the free lymphoid mesenchymal reserves, particular mention should be made of the very large lymphocyte or hemocytoblast. This cell, which is mobilized from fixed reticular cells and by mitotic proliferation, frequently appears in great numbers during severe infections and after immunization procedures. It suffers from many aliases, such as acute splenic tumor cell, myeloblast, lymphoblastic plasma cell, plasmablast, developing or immature plasma cell, macrohistiocyte and basophil macrophage. Our own investigations indicate

that it is a primitive lymphoid cell with marked mesenchymal potencies and possesses all of the developmental potencies indicated by these names. In chickens with their markedly active connective tissue, it becomes the chief lymphoblast, hemocytoblast, erythroblast and plasmablast of the spleen and bone marrow during severe malaria (W. H. and L. G. Taliaferro, 1955). Its role of plasmablast is of great interest because of the recent evidence that it and/or other stages of the plasma cell series produce antibodies (Coons *et al.*, 1955, Leduc *et al.*, 1955, and review by Taliaferro, 1949b).

ANTIBODY FORMATION

For technical reasons, we turned to the use of hemolysins produced in the rabbit to study the antibody-forming capacity of the spleen. This system has the advantage that the rabbit is large enough to make frequent and long-continued bleedings, and titrations of hemolysin can be made by the highly sensitive 50 per cent endpoint with modern photoelectric colorimetric methods. I should like to stress that this work involves the intravenous injection of whole sheep red cells or of boiled sheep red cell stromata. Whole sheep red cells contain heat-stable Forssman antigens as well as the heat-labile sheep isophile antigens, whereas heated stromata contain the Forssman antigens alone. Both give about the same results. When the antigen is introduced intravenously, the spleen plays a more prominent role in antibody formation than when introduced intraperitoneally or in the foot pad.

After a single injection of whole sheep red cells, a fairly stereotyped rise and fall of antibody occurs in the serum of rabbits (Fig. 5). Following the induction period, the first antibody rise is rapid and may take place at a single rate or in two phases. Peak titer is immediately followed by a comparatively rapid decrease in antibody for a period of approximately 3 weeks and then by a plateau or gradual decrease in antibody over a long period. Mathematically, such a curve permits a number of estimations regarding antibody formation. As may be noted in Figure 5, the data points fall remarkably well on a discontinuous curve, each segment of which is described by the well-known compound interest differential equation

$$\frac{dA}{dt} = k_1 A \tag{1}$$

or in integrated form

$$A_0 = A_t e^{k_1 t} \tag{2}$$

in which A_0 and A_t represent serum antibody at times 0 and t, respectively, and k_1 is the rate constant of antibody change (W. H. and L. G. Taliaferro, 1950).

The hemolysin curve is essentially the same when repeated injections of antigen are given from 3 to 8 weeks (Fig. 6, solid line) except that peak titer reaches a value approximately one log higher, the fall from peak titer is more rapid and prolonged, and the data points do not fit the theoretical curve as well (W. H. and L. G. Taliaferro, 1951b).

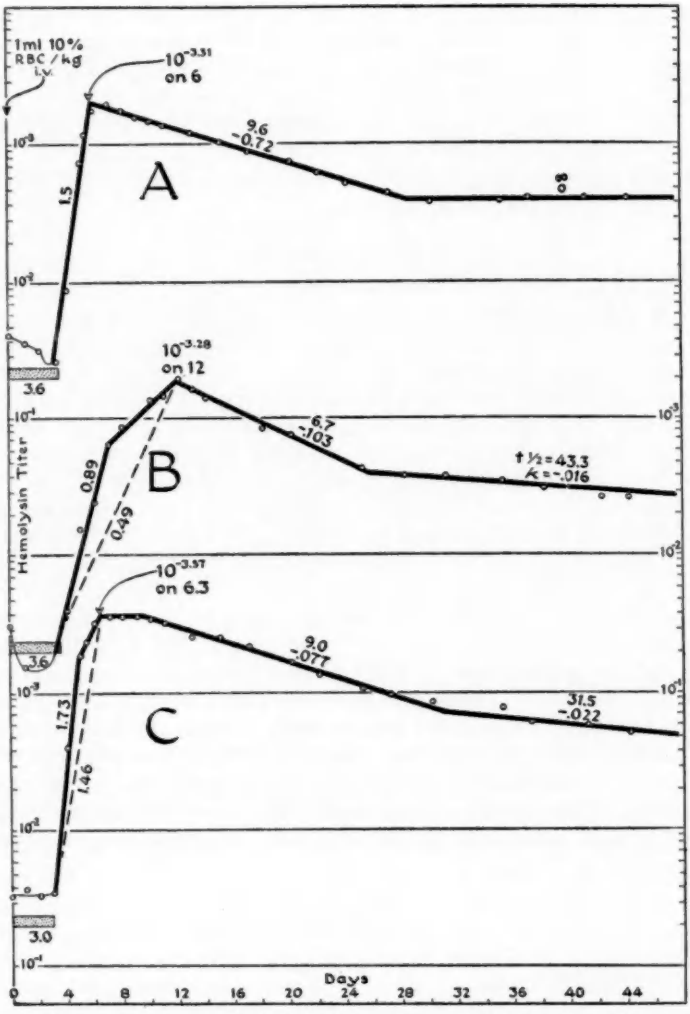

FIG. 5. Changes in serum antibody in 3 rabbits after a single injection of sheep red cells. The rate constants are shown by the values of k_1 and, when these are negative, the apparent half-life is given in days above the rate constant. (From Taliaferro, 1955.)

In analyzing the hemolysin response, we have attempted to measure the amount of antibody formed and the rate of the immune process in general. Serum antibody represented in Equation 1 is one aspect of antibody formation and results from at least two equilibria. In part, it is the result of the concentration equilibrium between tissues and blood. In part, it is the net antibody, i.e., the

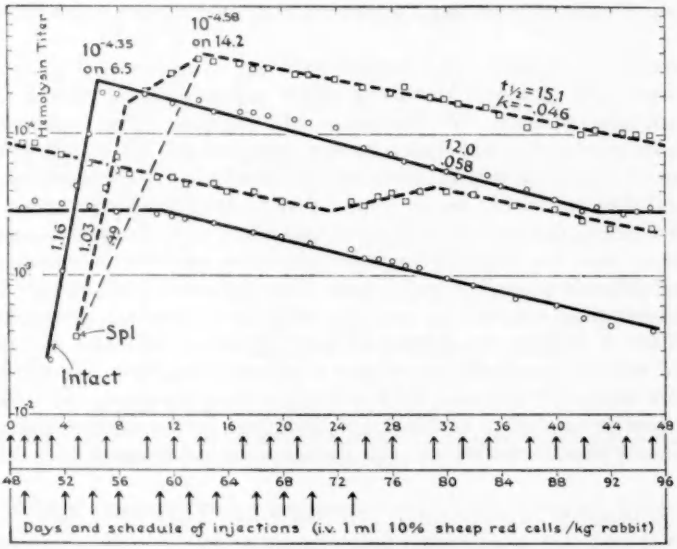

FIG. 6. Changes in serum antibody in typical intact (solid line) and splenectomized (broken line) rabbits following repeated injections of sheep red cells. Note that the splenectomized animal started to form antibody later, but attained 1.7 times the peak titer of the intact animal. (From Taliaferro, 1955.)

total amount formed minus the amount destroyed as a result of its continuing metabolic decay. At present we cannot calculate with accuracy the proportion of antibody in the tissues as has been done with certain other rabbit antibodies (Taliaferro, 1955). We can, however, approximate fairly well the amount of serum antibody that decays. As this decay is always dependent upon the amount of antibody present, it can be expressed by the differential equation

$$\frac{dB}{dt} = k_2 A \tag{3}$$

in which B is the amount of antibody which decays and k_2 is the rate constant of decay.

The following differential equation (Taliaferro, 1955) gives the total serum antibody formed, i.e., the antibody in the serum (A) plus that which has decayed (B).

$$\frac{dS}{dt} = (k_1 + k_2)A \qquad \text{(from Equations 1 and 3)} \tag{4}$$

or in integrated form

$$S_t = A_0 \frac{k_1 + k_2}{k_1} (e^{k_1 t} - 1) \tag{5}$$

in which S represents the total amount of serum antibody formed (Taliaferro 1955).

In the above equation, we determined the rate of metabolic degradation by ascertaining the biological half-life of serum antibody during passive transfer in a nonimmune rabbit. We (Taliaferro and Talmage, 1956) found that the Forssman and isophile hemolysins have a biological half-life of approximately 2.8 days. Actually, no isophile hemolysin is formed after single injections of red cells and does not appear regularly until 4 weeks of multiple injections.

In estimating the antibody output of the spleen as contrasted to that from nonsplenic sites, we originally assumed that there was little or no interaction between different antibody-forming sites. Thus, the amount of antibody formed by splenectomized animals was used as a measure of nonsplenic production and the difference between the amount of antibody formed in intact and splenectomized animals was used as a measure of splenic production. We still believe that this method is adequate after a single antigen injection, but we had to devise another method to analyze multiple antigen injections after finding that interactions between the organs occur during prolonged immunization.

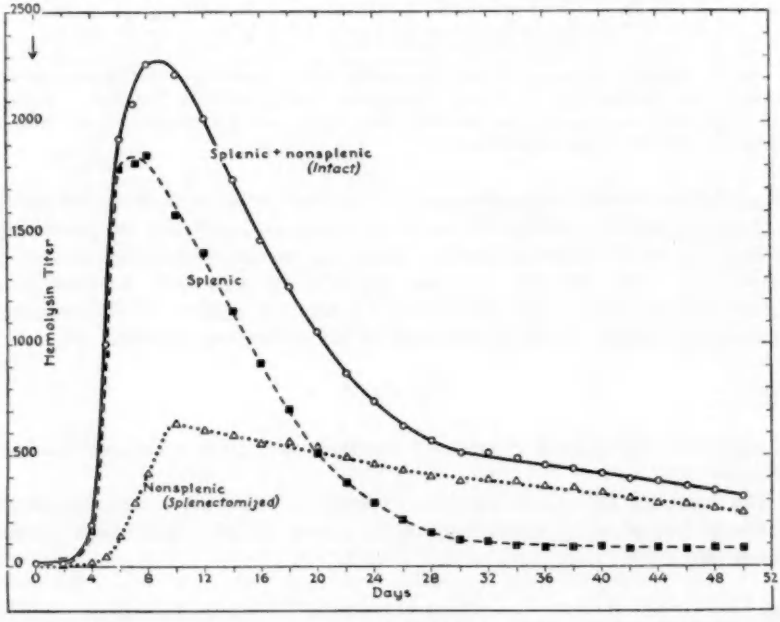

FIG. 7. Mean hemolysin curves after a single injection of sheep red cells for 18 intact rabbits (splenic + nonsplenic), 14 splenectomized rabbits (nonsplenic), and the difference between their individual mean titers (splenic). The titers of individual animals were taken from smoothed curves such as shown in Figure 5. (From data of W. H. and L. G. Taliaferro, 1950.)

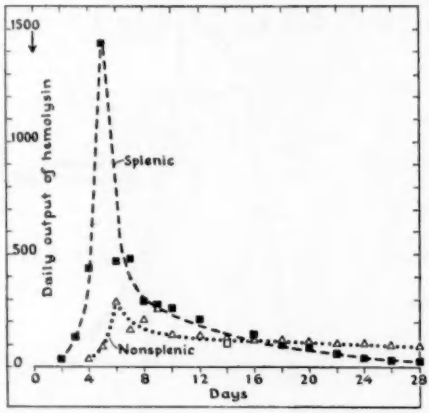

FIG. 8. Calculated total daily hemolysin output of the spleen and nonsplenic sites following a single antigen injection (Taliaferro, 1955).

A division of antibody into splenic and nonsplenic was obtained as follows. Hemolysin titers were determined and plotted every two or three days in 18 intact animals and in 14 splenectomized animals following the intravenous injection of one dose of whole sheep red cells. Differences between the two groups were also obtained. Thus, the three curves in Figure 7 give, respectively, the changes in serum antibody from splenic and nonsplenic sources (intact animals), from nonsplenic sources (splenectomized animals), and from the spleen (intact animals minus splenectomized animals). We have used the values in Figure 7 in conjunction with Equation 5 to calculate the total daily antibody output of the spleen (the serum antibody + the antibody that has decayed). These data (Fig. 7 and 8) clearly indicate that the spleen pours out a large quantity of antibody for several days but that its output then rapidly drops below the level adequate to replace metabolic decay. Nonsplenic sources, on the other hand, form antibody more slowly but continue to produce a fairly constant amount over a long period. Thus, the spleen forms as much antibody in about a week as all the rest of the sources form in 3 weeks (Taliaferro, 1950).

Antigenic stimulation during infections resembles more closely the situation in which antigen is injected repeatedly than that when it is injected just once. Multiple injections of antigen gave some surprising results. The most unexpected result was that splenectomized rabbits usually form more antibody than intact ones (Fig. 6 and 9). Thus, in repeatedly injected rabbits, the spleen is at first an asset but later is a liability in that it inhibits the formation of antibody by nonsplenic sites (W. H. and L. G. Taliaferro, 1951b, and Taliaferro, 1955).

To ascertain the fraction of serum antibody formed by the spleen in repeatedly injected rabbits, we carried out the following procedure. We subtracted the titers of the splenectomized group from those of the intact group through the 7th day when the greatest difference (6560 units/ml. serum) occurred. These amounts

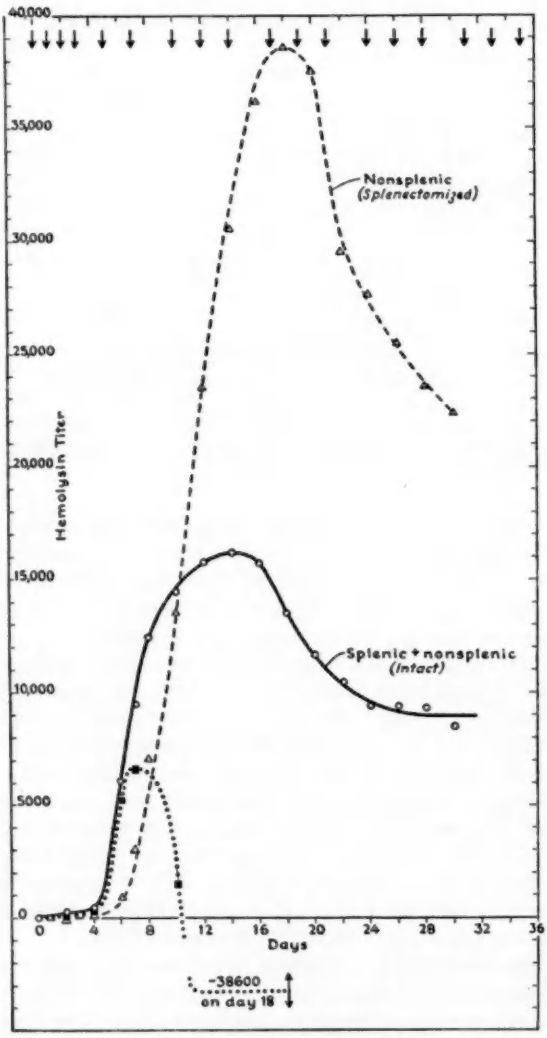

FIG. 9. Mean hemolysin curves during repeated antigen injections for 14 intact rabbits (splenic + nonsplenic), 11 splenectomized rabbits (nonsplenic) and the differences between their mean titers (splenic). Note that the "splenic" curve becomes negative shortly after the 7th day. The titers of individual animals were taken from smoothed curves such as shown in Figure 6. (From data of W. H. and L. G. Taliaferro, 1951.)

were fairly accurate because very little antibody was formed in the splenectomized rabbits during the first week. From the 8th day on, however, when large amounts of antibody occurred in the splenectomized animals (Fig. 9), an independent method of determining the capacity of the spleen was used. It was

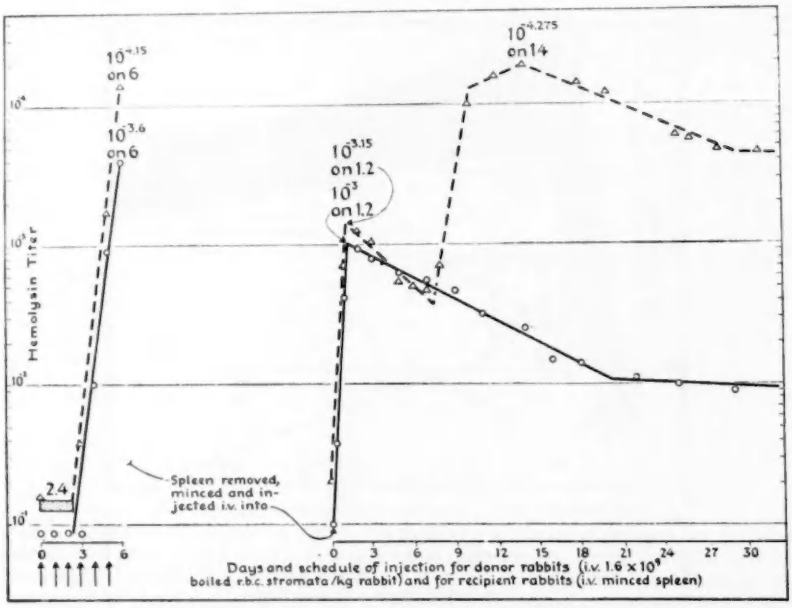
$10^{4.275}$ on 14
$10^{4.15}$ on 6
$10^{3.6}$ on 6
$10^{3.15}$ on 1.2
10^{3} on 1.2
Hemolysin Titer
Spleen removed, minced and injected i.v. into
2.4
Days and schedule of injection for donor rabbits (i.v. 1.6×10^9 boiled r.b.c. stromata/kg rabbit) and for recipient rabbits (i.v. minced spleen)

FIG. 10. This graph exemplifies the spleen transfer procedure. (From Taliaferro, 1955)

based on a method first used by Deutsch in 1899. This method consists of transferring spleen mince intravenously from an immunized donor to a normal (or X-rayed) recipient. The transferred cells or enzymes from a properly prepared donor then almost immediately begin to pour antibody into the recipient's serum which reaches a peak titer in a day or two (Fig. 10). (A subsequent rise in antibody may occur which is due to the injection of antigen present in the donor spleen.) The early peak titer in such recipients can thus be used as the relative capacity of the corresponding donor spleen to form antibody on the day of transfer. The mean values of the antibody-producing capacity of the spleen (Fig. 11) can then be added each day, after correcting the value of each previous amount for metabolic decay, to give an integral curve of splenic antibody. The data from such calculations were used from the 8th day on after superimposing the actual value (660 units/ml. serum) obtained by this method for the 7th day on the greatest difference (6560 units/ml. serum) obtained in Figure 9. The discrepancy between these two values could be associated with the crude method of spleen mince transfer.

Figure 12 shows the results of these calculations. The curve for intact animals is based on actual experimental data. The curve for nonsplenic sources is based through the 7th day on actual experimental data and from the 7th day on is the difference between the amount formed in the intact animal and the calculated amount formed by the spleen. These daily outputs are expressed arithmetically in Figure 13. It may be seen that here again the spleen forms antibody sooner

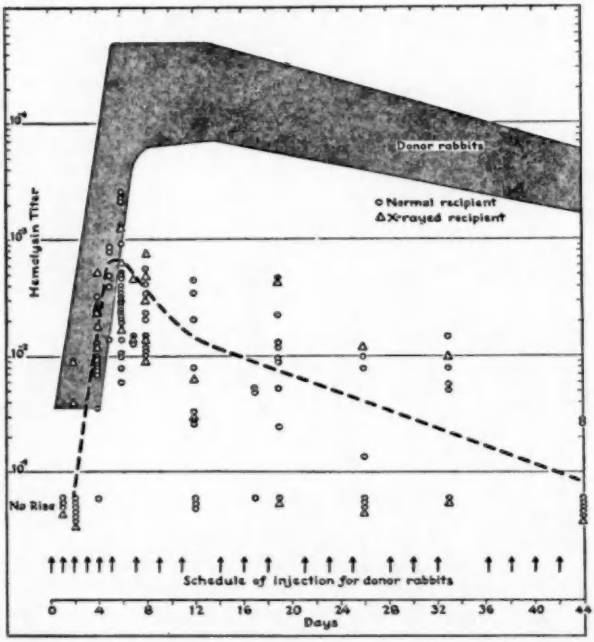

FIG. 11. Changes in the antibody-producing capacity of spleen mince (broken lines) and the range in peak titer in individual recipients (circles for normal and triangles for previously X-rayed) during the rise and fall of antibody in donor rabbits (shaded area). The peak titer of each recipient is placed on the day on which the donor spleen was removed. (From Taliaferro, 1955.)

and attains a higher peak output than nonsplenic sources. Nonsplenic sources, however, form more antibody than the spleen because they continue for a much longer time. In addition, nonsplenic sources form relatively much more antibody after repeated than after a single injection of antigen (Taliaferro, 1955).

I realize that our conclusions are based on certain assumptions and extrapolations. Nevertheless the data indicate after both single and repeated antigen injections that the spleen forms a large amount of antibody initially and then rapidly stops forming antibody, whereas nonsplenic sources do not even begin to form antibody until the spleen is producing antibody at a high rate but then continue to form antibody at a lower rate over a longer period. The spleen probably is critical in the early response to an infection, but is soon surpassed by other sites in the maintenance of antibody levels over long periods.

The cessation of antibody formation by the spleen has a different basis after single and after multiple antigen injections. Mrs. Taliaferro and I (1952) found that reinjecting an animal after a single injection of antigen, probably at any time after initial peak titer, results in the formation of antibody in an amount

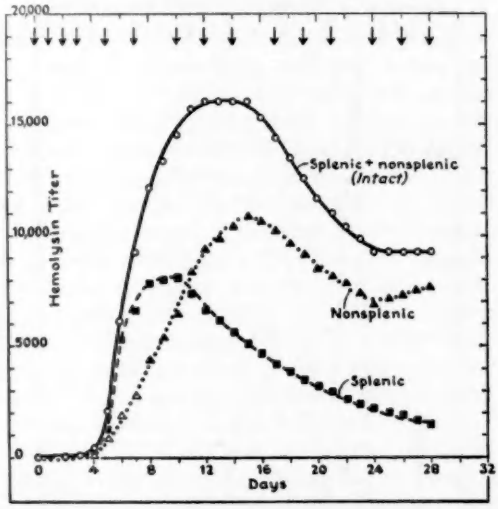

FIG. 12. Mean hemolysin curves during multiple injections of antigen for 14 intact rabbits (splenic + nonsplenic), 11 splenectomized rabbits (nonsplenic), and the differences between their individual mean titers (splenic). Note that the curve for splenic activity was obtained from actual experimental data for 7 days and calculated for the remaining 21 days as described in the text. The curve for the intact animals is identical with that shown in Figure 9. (From data of W. H. and L. G. Taliaferro, 1951.)

statistically similar to that formed during the initial response. This finding indicates strongly that the inactive phase of the spleen after a single injection of antigen is due to the lack of antigenic stimulation. Such a lack of antigenic stimulation does not play a role in the cessation of antibody formation after multiple injections because antibody declines while antigen injections are being continued. We do not know the basis for this refractory phase but it is independent of any absorption of antibody from the serum by the injected antigen because of results obtained on the transfer of splenic mince from repeatedly injected donors (Fig. 11). It is probably not the result of reticulo-endothelial blockade, as usually conceived, because removal of the spleen increases antibody formation instead of inhibiting it as is generally true with ordinary blockade when the function of macrophages is depressed by the repeated injection of particulate matter. We do not know its relation to Felton's (1949) paralysis of the immune mechanism following the accumulation of carbohydrate antigens in the body.

Whatever the mechanism leading to the inhibition of antibody formation during repeated antigen injections, it seems to be associated with some type of interorgan regulatory process. Thus, in unpublished work, Mrs. Taliaferro and I have found that after an animal is in a refractory stage and neither the spleen nor other sites are forming much antibody, splenectomy in conjunction with further antigen injections is followed by a resumption of antibody formation.

We have provisionally lumped the nonsplenic antibody sites into one category

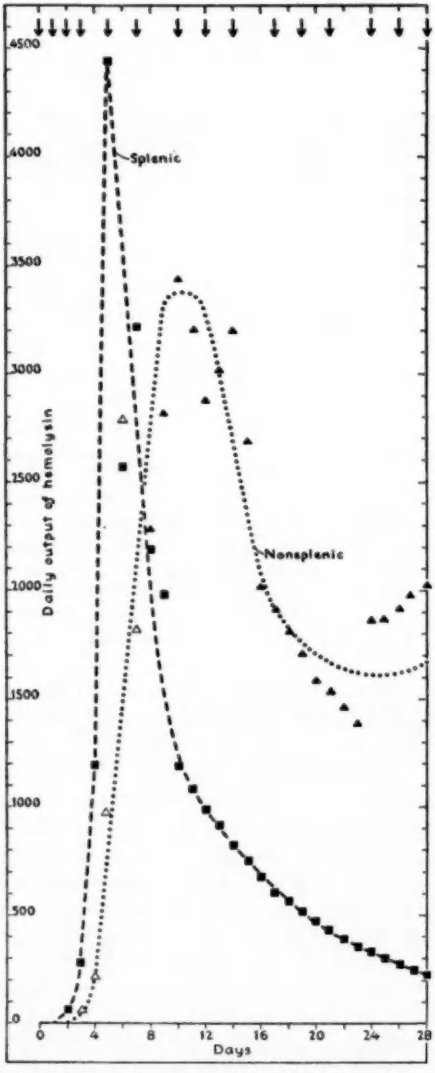

FIG. 13. Calculated total daily hemolysin output of the spleen and nonsplenic sites during repeated antigen injections. (From Taliaferro, 1955.)

until more is known concerning them. It is reasonable to assume, however, that different nonsplenic sites behave somewhat like the spleen and stop forming antibody at different intervals and thus cause step-like decreases in the serum antibody (see discussion by W. H. and L. G. Taliaferro, 1950).

ANTIBODY SYNTHESIS

At the present time we have evidence that antibodies arising in the same animal may differ in their physical characteristics, such as molecular weight and metabolic half life, and in the fraction of globulin in which they appear. On the other hand, we have no evidence that they differ from the other globulins of the fraction in which they occur except in their specific reactivity to their homologous antigen. Their characteristic specific reactivity is almost certainly due to some modification in the surface characteristics of the globulin molecule. One of the central problems in antibody formation, therefore, is: what leads to the synthesis of an entirely new molecule with these changed surface characteristics?

Studies on the function of the spleen in antibody formation together with work on the effects of X irradiation have given us some leads in connection with this basic problem. The pioneer experiments of Benjamin and Sluka (1908) indicated that heavy doses of X irradiation given to the whole body prevent antibody formation—particularly if the antigen is injected after irradiation. In recent years, Kohn (1951) and especially Dixon and his co-workers (1952) and we (Taliaferro *et al.*, 1952, and W. H. and L. G. Taliaferro, 1954b) have shown that this type of inhibition to antibody formation is due to a very short, possibly almost instantaneous, radiosensitive event early in antibody formation. Once this period is passed, the administration of even lethal doses of X rays is generally followed by the production of normal or above normal amounts of antibody provided the animal lives long enough. An understanding of what occurs during this short radiosensitive period might clarify not only the mechanism of X-ray inhibition to antibody formation but also the more fundamental question of what initiates the synthesis of a new globulin with the reactivity of antibody.

Beginning in 1952, Harris and his co-workers showed that the anergic X-rayed animal would form agglutinins when injected with antigen and cells from lymph nodes—either separately or as a mixture. Jaroslow and I (Jaroslow *et al.*, 1954; and Jaroslow and Taliaferro 1955a and b) have obtained similar results with hemolysin using spleen mince. Pursuing this problem further, we ascertained that the restoration of antibody formation in an X-rayed animal is not dependent upon homologous or intact cells. It takes place when we use mouse spleen, the HeLa tissue culture strain of cells derived from a human cervical carcinoma, the supernatant from ground up HeLa cells or a yeast autolysate after centrifugation in high gravitational fields. We do not yet know the real basis for this restorative action on antibody formation, but my personal guess is that it is some enzymatic or other action on the antigen which is an initial necessary step in antibody formation.

After this initial event is passed, the induction period occurs. Surprisingly little attention has been paid to what happens during this time. Recently, Green and Anker (1954) found that different isotopically labeled amino acids when administered during the induction period of a secondary response of the rabbit to ovalbumin appeared in blood taken on the 5th day. They came to the conclusion that about 31 per cent of the amino acids in the antibody were drawn from the amino acid pool during the induction period. We (Taliaferro and Talmage, 1955)

reached another conclusion using the technique of spleen transfer (cf. Fig. 10) and the secondary response in rabbits to bovine serum albumin. Briefly stated, donor rabbits were given large quantities of yeast hydrolysate containing radioactive S^{35}-labeled amino acids during the induction period, and the spleen, removed just before precipitating antibody appeared in the donor serum, was minced and transferred to a nonimmunized recipient. As a result of this procedure, antibody appears almost immediately in the recipient. In spite of the fact that the donor produces highly labeled antibody, there is extremely little labeling of the antibody in the recipient. These experiments strongly indicate that not more than 1 per cent and probably only about 0.03 per cent of the amino acids occurring in antibody is drawn from the amino acid pool during the induction period. Although comparatively little antibody was formed in the recipient, amino acids were incorporated at the same rate as in the donor. We believe that the induction period is probably not a period of the synthesis of amino-acid-containing antibody precursors, but is a period during which new enzymes are developed which in turn synthesize antibody. We further believe that Green and Anker's results were largely due to the labeling of complement which was later carried down with the antigen-antibody precipitate. Our method of transferring the synthetic mechanism from a labeled donor to an unlabeled recipient avoided any marked contamination of the immune precipitate with such labeled complement.

My talk today has touched on many facets of the spleen as an agent in immunity. This organ possesses a rare combination of phagocytic, cytopoietic and antibody-forming capacities essential in immunity, and provides excellent material for varied types of immunological research.

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THE CONFUSING EPIDEMIOLOGY OF MALARIA IN CALIFORNIA[1]

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Some fifteen years ago I wrote (Herms and Gray, 1940) that in any long existing situation a sceptical attitude may be valuable, for it is possible that an idea which has stood unchallenged for a generation may be wrong at least in part, not having kept pace with the accretions of new information.

Since at least 1910 it has been generally held that *Anopheles freeborni* was *the* vector of malaria in California, and that other anophelines had slight if any part in the chain of transmission. On the basis of the malaria then existing in California it appeared to be the principal if not the only effective vector. But in recent years more complete information as to the early history of the disease in the State, together with numerous observations by many workers in the field of mosquito control concerning the habits of the anophelines in California, as well as some recent outbreaks of malaria which differed in pattern from previous epidemic situations, have thrown doubt on the role of *A. freeborni* as the principal vector. It now appears probable that *A. punctipennis* may play a larger part in the transmission cycle.

We do not know precisely when or by whom malaria was introduced into California. It was probably introduced, and perhaps re-introduced, from the lower Columbia and Willamette River valleys in Oregon by fur brigades of the Hudson's Bay Company, by explorers, and later by settlers.

We can assume that at least four species of *Anopheles* have been present for many centuries in California prior to its discovery by the white man. They are *A. freeborni*, *A. punctipennis*, *A. franciscanus* and *A. occidentalis*. On the basis of considerable negative evidence we can assume that the *Plasmodium* of malaria had not been introduced into the indigenous Indian population prior to the advent of the white man. Until recently it appeared that malaria probably was introduced from Oregon in about 1843, but Cook (1955) has now indicated that malaria may have first appeared among the Indians of the upper Sacramento Valley in the summer of 1829, via trappers of the Hudson's Bay Company from Oregon. The disease appears to have spread southerly, slowly at first, and then to have resulted in a tremendous epidemic which killed off a very large part of the Indians in the great central valley of the State in 1833. Cook has assembled a large amount of information which in spite of some discrepancies and doubts is very plausible. He estimates the mortality rate to have been at least 75 per cent, probably higher. This was about the same rate that occurred in the valleys of the Willammette and lower Columbia Rivers in Oregon in the same years.

There may be some doubt that an epidemic with this mortality could have been malaria in the California valleys, but there is no doubt that it was malaria in

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Oregon, aided probably by various other white man's diseases, and in spite of some inconsistencies in the reports of early observers, the evidence seems to be strongly in favor of an epidemic of malaria in California in 1833, with a high mortality rate which greatly reduced the indigenous Indian population in the central valley. It did not affect the Spanish speaking settlers nor the Indians in the coastal areas and in southern California.

Subsequent to 1833 little is known about malaria in the State. John Marsh, who lived on the western edge of the valley, is reported to have treated the Bolgones tribe of Indians for malaria in 1837. Except for a few roving bands of trappers, and occasional forays from the coastal haciendas by rancheros in pursuit of stolen horses, there were practically no white persons in the valley until, in 1839, John A. Sutter established his fort (the first permanent white settlement in the valley) at the confluence of the Sacramento and American Rivers, and began the development and settling of the Sacramento Valley. There is no evidence that the rancheros who went into the valley brought back malaria with them to the coastal region, and if the disease was prevalent it would seem that surely some of them would have been infected.

From the establishment of Sutter's fort in 1839 until the "gold rush" of 1849 we could for a long time find no evidence of malaria at the fort or at the several ranches which he and John Bidwell established as far north as the present Chico area in Butte County. During this period hundreds of people arrived in California overland, many of them via Sutter's fort, thence taking up land in the valley. Among these there were almost certainly latent infections, coming, as many of them did, from malarious areas in the Middle West. Recently R. E. Fontaine of the California State Department of Public Health, who is collaborating with me in the writing of a more complete history of malaria in California, has found statements in the "New Helvetia Diary" which was kept at Sutter's fort, and also in Lienhard's recollections entitled "A pioneer at Sutter's fort", that chills and fever were prevalent among both whites and Indians at the fort, and among the settlers in the valley, during the summers of 1846 and 1847. So far we have found no information on malaria at the fort prior to 1846.

With the discovery of gold in 1848 at Coloma near Sacramento, the great migration called the "gold rush" began across the continent by wagon trains and by ship via Panama or Cape Horn. Most of the gold seekers went into the placer diggings in the foothills, on the lower courses of the numerous tributaries of the two main rivers, and malaria became such a scourge of the gold camps that it was frequently referred to as "miner's fever". Apparently *vivax*, *falciparum* and *malariae* infections were present, with a substantial percentage of *falciparum* as a result of infections picked up on the Panama route.

By about 1856 placer mining in the lower foothill gulches began to decline, and vein or lode mining at higher elevations away from the stream beds increased. More people began to take up land on the floor of the valley and to go into agriculture. The available evidence, which is meager, seems to indicate that malaria declined, although still present, with a few local areas of relatively intense infection. This condition appears to have continued until about 1900, when irriga-

tion began to develop in the lower foothills of the Sierra Nevada mountains and on the valley floor. With irrigation, malaria again increased and by 1909 Dr. William F. Snow, then State Health Officer, termed it the "Minotaur" of California. New irrigation districts almost always resulted in a marked increase in malaria, as irrigation seepage was ideally suited to the production of *A. freeborni* in sunny exposures and of *A. punctipennis* in shaded areas.

Some of the more intense local endemic areas were controlled by mosquito abatement measures which began in 1910, but irrespective of these measures malaria began to decline in the central valleys after about 1920, even though in the Sacramento Valley the introduction of rice culture had resulted in a great increase of *A. freeborni*. Possibly improved housing, but more probably deviation to animals as a result of a marked increase in animal husbandry, may account in part for this reduction of the disease in the face of large increases of the apparent vector.

Except for a few local, relatively small epidemics, malaria practically disappeared from the central valley during the period from 1930 to the present, even though *A. freeborni* has remained very abundant in the Sacramento Valley and *A. punctipennis* has been abundant along the various streams in the foothills. Even the return of many infected ex-service men from World War II failed to revive indigenous malaria. Then in 1952 a sharp outbreak of malaria was set off at a girls' camp in Nevada County (Brunetti and Fritz, 1954) under conditions which brought into sharp relief the doubts of several observers as to the relative roles of the several *Anopheles* species in the State.

At about the time (1910) when malaria began to be studied by W. B. Herms, W. F. Snow, W. A. Sawyer and others, the conditions of irrigation were such that there was little doubt in the minds of competent observers that *A. freeborni* (at that time called *A. quadrimaculatus* and later *A. maculipennis* (Aitken, 1945)) was the effective and apparently the only vector. Under the type of irrigation then practiced, with leaky unlined canals and ditches in earth, and with the resulting wisespread shallow seepage areas exposed to sunlight, *A. freeborni* was the dominant *Anopheles* on the valley floor in the late summer and early autumn months when the greatest incidence of malaria occurred. *A. punctipennis* was more prevalent in the spring and early summer, and generally was confined in its larval habitat to the shaded marginal pools in the streambeds of the valley and up into the foothills. Furthermore, *A. freeborni* was frequently found in houses, whereas *A. punctipennis* seldom entered houses, although it bit freely outdoors in the evenings and in shaded areas in the daytime. We called it a "porch biter". This failure of *A. punctipennis* to enter houses was one of the reasons it was dismissed as an appreciable vector.

Actually, however, a more careful appraisal of the habits of the people living in the central valley should have indicated the possibility that *A. punctipennis* could have been an effective and frequent vector. The daily temperatures in the central valley are high and the nights hot until near midnight from mid-June until mid-September. Since there was no air conditioning for home cooling in those days, it was common practice for people to sit outdoors in the evenings until

the houses cooled down sufficiently for comfortable sleep, and many people slept outdoors or on porches, usually without screens or bed nets. Under such conditions *A. punctipennis* was at least as apt to attack as *A. freeborni*.

Unfortunately there was never enough manpower, funds or time available in those early days to make an adequate infectivity study of the *Anopheles* species in an endemic area. The record of the few dissections I was able to make in the Penryn area in 1910 have been lost, but my recollection is that they were inconclusive. No one, to my knowledge, has given this part of the problem any extensive study. Simmons and Aitken (1942) said that further studies of *A. punctipennis* "will be required to determine more exactly its relative importance in the different regions where it exists." This lack represents a definite gap in our knowledge of malaria transmission from 1910 to the present. Even when in 1952 an epidemic of malaria occurred at Lake Vera in Nevada County it was not possible to make a study of the mosquito infectivity rates, as all available manpower was concentrated on the rather severe epidemic of Western equine and St. Louis encephalitis that year. Furthermore, it is doubtful if much could have been learned, as it was about a month after the time of infection that the then widely scattered cases could be traced to a common origin.

However, the Lake Vera outbreak set several of us, particularly R. E. Fontaine and myself, to thinking again about this problem of vectors. As the group of girls encamped at Lake Vera generally slept in tents or in only partially screened buildings, or even outdoors, they were perhaps more exposed to bites by *A. punctipennis* than by *A. freeborni*, in view of the rather well observed habits of the two species. Also, *A. punctipennis* was numerically the more prevalent species based on adult captures. However, numerical percentages are not necessarily indicative of the relative importance of *Anopheles* species as vectors.

I also remembered an outbreak of malaria near Lodi in San Joaquin County in 1934, when 69 cases were reported from that area, mainly among a migratory population camped along the bottoms of the Mokelumne River. Many of these migrants came from Oklahoma and Texas, and may be presumed to have had latent infections, but the type of outbreak appears to have included many new infections as well as relapses from previous infections. The interesting feature of the outbreak was that it occurred under conditions where *A. punctipennis* was the dominant *Anopheles*, and under conditions where it could attack freely and persistently.

This again led to more thinking about malaria in the "gold rush" period from about 1849 to the middle 1850's. Most of the mines in that period were concentrated along the lower courses of the rivers, in foothill areas where *A. punctipennis* is today and to our knowledge has been since 1910 more prevalent than *A. freeborni*. The miners, also, lived in tents or under crude housing conditions such as lean-to's or cabins, or even in the open, where they would be freely exposed to attack by *A. punctipennis* as well as by *A. freeborni*.

The early California Indians also lived mainly along the rivers and streams, where even today *A. punctipennis* is generally more prevalent than *A. freeborni*,

and their habits of living were such that they would be well exposed to attack by *A. punctipennis*.

The picture is much less clear on the floor of the valley away from the streams, after agriculture was introduced and particularly after the introduction of irrigation about the turn of the century. The early canals were unlined, and there was extensive lateral seepage from many of these unlined canals and ditches. This shallow seepage, exposed to sunlight, is the preferred larval habitat of *A. freeborni*, and this species developed in large numbers, and malaria was a constant accompaniment of new irrigation projects. As these canals and ditches silted up and leaked less, and as the demand for water increased, leading to the lining of canals with concrete, or the use of pipe lines, the seepage was reduced. This was especially noticed in the southern part of the valley, the San Joaquin Valley, where water for irrigation was less available than in the Sacramento Valley. To the south malaria declined more rapidly and disappeared earlier than in the Sacramento Valley to the north. In a few irrigation projects, such as Los Molinos in Tehama County, which were intersected by small, shaded streams, *A. punctipennis* remained high numerically and on a percentage basis.

It does not appear that gold dredging in the stream beds where the rivers debouched from the foothills, or hydraulic mining in the foothills and lower mountains in themselves made much difference in malaria prevalence. Both operations muddied the waters so much as to be a deterrent to both species of *Anopheles*. But the leaky canals and flumes bringing water to the hydraulic monitors furnished extensive hillside seepage areas particularly adapted to *A. freeborni* where exposed to sunlight. I well remember a hillside seepage from a ditch near the ridge town of North Columbia in Nevada County in 1918. This was the source of the *Anopheles* maintaining an endemic malaria in that small community. Since no one would do much more than lend a shovel, I dug a few ditches, whereupon malaria ceased in North Columbia. In those days a State District Health Officer had to do everything, from digging ditches and capturing rabid coyotes, to vaccinating for smallpox and examining prostitutes.

About 1915 rice began to be grown in the Sacramento Valley, and today is an important crop. At first *A. freeborni* was not very prevalent in the rice areas, but today it is present in very large numbers in many, though not all, rice fields in the State. At first the crop was grown largely with Hindu labor, but today it is a mechanized industry using little manpower. Malaria has not been a problem in the rice areas, in spite of the swarms of *A. freeborni*. Possibly this has been due in part to the very small human population actually residing in the rice field areas; both operators and laborers live in the adjacent towns. Another factor may be, as observed by R. E. Fontaine (personal communication) that in the hot months of July and August *A. freeborni* does not appear to bite humans, though in its migratory flight in mid-October it is a fiercely biting pest, as it is also in broad daylight on warm days in January in towns near the rice areas. *A. punctipennis* is practically absent in the rice areas.

It is interesting to note in passing the observations of Stage and Gjullin (1935)

that *A. punctipennis* is numerically preponderant in the Willamette and lower Columbia River valleys in Oregon, where malaria has ranged from severe epidemic to mildly endemic prevalence, and that *A. freeborni* is numerically preponderant in eastern Oregon and Washington, where malaria is practically absent.

A few comments on environmental conditions in the central valley and in the Sierra Nevada foothills of California, and the changes in these conditions, may be of interest. There is no reason to believe that the climate of California has changed enough in the past 125 years to have had any effect upon the mosquito population or on the transmissibility of the *Plasmodium*. The other conditions which have markedly changed are the introduction of irrigation; water storage and river regulation; urbanization and the location of towns; animal husbandry; housing; and illumination at night.

Irrigation has definitely been a factor of importance. In many places its introduction has been accompanied by an increase in malaria under conditions where *A. freeborni* appears to have been the more probable vector. But in spite of a great increase in irrigated lands in recent years malaria has died out in the presence of very large numbers of *A. freeborni*.

Irrigation districts, cities, power companies, the State and the federal government have constructed many dams and reservoirs in the mountains and in the foothills. Since the ecology of the California anophelines is markedly different from that of *A. quadrimaculatus* in the southern states, these reservoirs have had no effect on the *Anopheles* population or malaria in California. The changes in river flow resulting from these impoundments have somewhat reduced extensive flooding of the valley during the winter and spring runoffs, and in some places have maintained a more uniform summer flow in the rivers. Neither factor appears to have had an appreciable or uniform influence on the prevalence of any *Anopheles* species in the summer or early autumn when malaria was at its peak.

While the rural agricultural population has increased, the trend has been toward urbanization. Many of the cities and towns have been located away from the rivers and streams, and those adjacent to the main rivers are usually on the lower reaches where conditions for the development of either *A. freeborni* or *A. punctipennis* are not favorable. In some cases the discharge of sewage from cities into the adjacent streams has been an adverse influence on the anophelines.

Increased animal husbandry may have had a definite influence. Prior to the advent of the white man, the central valley and the foothills teemed with a large wild animal population on which, no doubt, the mosquitoes fed well, and also on the indigenous Indians. The white man and his works exterminated these animals, or greatly reduced them—the tule elk, deer, bears, rabbits, predators, aquatic wildfowl, etc. The white man also ruthlessly exterminated the Indians. There was a period of from, say, about 1870 until about 1920 when deviation to animals was probably slight. Since then animal husbandry has greatly increased in the central valley. But we do not know the host preferences of the California *Anopheles*. Adequate studies of blooded females in nature might show whether

the present preferences of the several species are for human, bovine or avian blood, but this would not be conclusive as to past decades.

Improved housing in recent years may have had an effect on transmission by *A. freeborni* but probably not on transmission by *A. punctipennis*. The tendency of Californians to live outdoors, especially on summer evenings, might somewhat offset any effect of better housing.

The astonishing increase in illumination at night of cities and homes in the past fifty years might be a factor. It might have a tendency to discourage attacks by *A. freeborni* but to have a relatively less deterrent effect upon *A. punctipennis*.

At least until the past few years medical treatment could have had little effect or no effect upon the transmission of infection, and whatever effect it might have had would have been the same upon any *Anopheles* species.

Whether these changes in environment have been a major factor in the reduction of malaria in the central valley, and whether they operated differentially on the two principal species of *Anopheles*, will be an interesting study. Possibly the decline of malaria in the upper Mississippi Valley as described by Ackernecht (1945) may have a parallel here. Certainly *A. quadrimaculatus* and *A. punctipennis* are still prevalent in that region, but malaria has disappeared. Time does not permit me to pursue this interesting matter further in this address.

If *A. freeborni* has been the principal vector of malaria in California, it is difficult to understand the almost complete absence of the disease on the floor of the valley in spite of an obvious great increase of *A. freeborni* in the rice field areas and the undoubted presence of many returned military personnel infected with malaria in foreign wars. With one exception that I can think of, at Winters in 1936, the few localized outbreaks in recent years have been either in the foothills or along river bottoms under conditions where *A. punctipennis* was more abundant than *A. freeborni*, or under conditions especially suited to attack by *A. punctipennis*.

Of necessity, because of time and space limits, a large amount of data has had to be omitted, but all of it tends to confirm our doubts and uncertainties as to which *Anopheles* has been the principal vector of malaria in California. There is lack of knowledge of both the mosquitoes and the *Plasmodium* prior to about 1910, and insufficient laboratory work since that time, to determine the relative infection rate in the mosquitoes in nature, or to determine host preferences, has deprived us of other sources of information. But what evidence is available appears to me to indicate that *A. punctipennis* may be a more important vector than opinion has heretofore held, and in the presence of malaria, measures to control *A. punctipennis* may be at least as important as measures to control *A. freeborni*.

In reviewing the history of malaria in California it appears to me that *A. punctipennis* probably was the principal vector in the great epidemic (if that was malaria) in 1830–33 among the native Indians, and that it was probably the principal vector in the mining camps during the "gold rush", say about 1849–56. On the other hand it is possible that in the early days of irrigation on the floor

of the central valley, from about 1900 to about 1920, *A. freeborni* may have had a primary part in the transmission of the disease, but with transmission by *A. punctipennis* probably also a factor.

There would be little point in bringing this relatively minor and local problem before this Society in a memorial lecture in honor of Charles Franklin Craig, were it not for the more general implications involved. Malaria is a subtle disease with infinite variations in its manifestations. The secrets of its transmission in particular regions have not been discovered easily, and discovery has sometimes progressed from error to error. The primary role of the relatively few *A. minimus* in South China, where *A. hyrcanus sinensis* is the predominant anopheline; the long failure to recognize *A. minimus flavirostris* as the primary vector in the Philippines; and the great difficulties in resolving the intricate *maculipennis* complex in Europe, are illustrations of the type of problems involved. I also feel that the apparent effect of changes in environment, which seem to have had an appreciable, though as yet imperfectly understood, part in the decline of this disease in the upper Mississippi Valley and in the central valley of California (and probably also in the Southern States) may be a basic concept which can be extended to the control of malaria in tropical areas. Some hard and pertinent thinking along these lines appears to be advisable.

Therefore, at least once in a generation we ought to re-examine our theories and our data in any epidemiological situation, especially any new information. By no means should we ignore those few inconvenient facts which do not fit into the established theories or conform to the general pattern of previous data. Possibly statisticians might prefer to toss them out as aberrations, but too frequently these aberrant items may be the keys which open the doors to correct knowledge.

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Papers Presented at

SYMPOSIUM ON NEWER KNOWLEDGE OF VIRAL AND RICKETTSIAL DISEASES

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INTRODUCTION

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In organizing a symposium the convener is almost invariably confronted with difficult decisions with respect to the subject matter to be encompassed. The difficulties of choosing specific facets of a problem or particular aspects of a relatively broad field are amplified when the symposium must, through temporal limitations, be confined to a matter of several hours rather than several days. In this afternoon's symposium, a comparatively broad coverage of the viral and rickettsial field might have been effected through discussion of the purification of viral and rickettsial agents, cellular metabolism and viral growth, the initiation of cellular infection by viruses and rickettsiae, the pathogenesis, immunity and epidemiology of viral and rickettsial infections, etc., and even of the fascinating subject of the role of the numerous unidentified viruses recovered from arthropods, birds and the human throat and intestinal tract in the causation of disease. Instead, this symposium is sharply restricted in scope to show (1) the tremendous impact of the new tissue culture methods on our approach to the study of the etiology, pathogenesis, epidemiology and control of viral diseases (2), that immunization with living attenuated poliomyelitis viruses is an attainable objective within the near future, and (3) that definite advances are being made in rickettsiology, a relatively neglected, albeit important, field.

While three of the four papers dealing with viruses may mention a number of diseases in passing, their underlying, unifying theme is the manner in which the new tissue culture methods permit cultivation in the laboratory of agents hitherto either not propagable or propagable only in unusual or expensive hosts. In the former case, viral diseases previously insusceptible of laboratory study are now amenable to such investigation; in the latter case, study is not only facilitated but can be conducted at a greater tempo. In both cases, it appears worth mentioning, the ability to cultivate and study an etiologic agent in the laboratory provides the means of endowing clinical and epidemiologic studies with a scientific precision not otherwise attainable, and the simpler the methods of detecting the

activity of that agent in a host or in a community, the greater the impetus to the study of that disease.

It was Enders, Weller and Robbins who worked out the tissue culture techniques that made possible for the first time the cultivation of the poliomyelitis viruses, a discovery whose far-reaching implications were recognized by a Nobel award to this team. Aside from the fact that the ability to cultivate poliomyelitis viruses in vitro has led to a resurgence of interest in poliomyelitis investigations, tissue culture techniques provide a fundamental weapon for an investigative attack upon diseases of known or presumed viral etiology, diseases upon which studies up to now have been either slow and unsatisfactory or entirely fruitless. The application of culture techniques, however, is beset with problems and pitfalls; Doctor Weller will discuss some of these.

The contributions of Doctor Syverton and his associates in the tissue culture field have also been of unquestioned value in advancing our knowledge of viral agents and diseases. The contributions of the Minnesota group have been in the direction of removing investigators from dependence upon human embryonic or simian tissues as a source of cells for the cultivation of viral agents. These investigators have worked out, and are constantly improving, methods for growing cells in continuous culture so that a constant supply of cells can be immediately and readily available for use. Indeed, much viral research today utilizes the methods developed by Doctor Syverton and his associates. Not only are HeLa cells used in studies on poliomyelitis, but they are used almost entirely for current research on viral respiratory diseases. Doctor Syverton will discuss the use of cells in continuous culture for the study of viruses, and Doctor Rowe will report on the findings of his group with the so-called "APC" viruses, a virus family in whose isolation and study the HeLa cell plays an invaluable role.

The family of viruses which Doctor Huebner has designated as the "APC" group, an abbreviated nomenclature from the designation "Adenoidal-Pharyngeal-Conjunctival," tissue sites from which these agents have been recovered, is proving to constitute an important group indeed. Doctor Wallace Rowe, of Doctor Huebner's laboratory, will discuss the clinical conditions with which these agents are associated, together with epidemiologic studies concerning these agents.

The fourth paper, that of Doctor Koprowski, deals not with tissue culture but with laboratory work in animals and with field tests in man. His paper is included as a representation of the opinion of that school of virologists who believe that safe, effective and long-lasting immunity against poliomyelitis (and other diseases as well) can better be achieved through vaccines composed of living virus whose pathogenic properties have been attenuated rather than through "killed" vaccines containing inactivated virus, virulent or avirulent. Doctor Koprowski presents the findings and observations of a study which has been under way for several years, proceeding carefully, undramatically and slowly in a logical step-by-step sequence.

This brings us now to the rickettsial diseases, a field which has been woefully and undeservedly neglected—so much so that the Armed Forces Epidemiological

Board has recently organized a Commission on Rickettsial Diseases to arouse and stimulate interest in them. Despite this neglect, a relatively small group of workers has added considerably to our knowledge although it is still true, as Doctor Smadel pointed out in a symposium before the Society of American Bacteriologists in 1950, that most of the advances are in practical aspects such as diagnosis, immunization, control and therapy and that abstract or, if you will, fundamental information on the nature of the rickettsial organism itself is still far more meager than that on viruses. Doctor Snyder will attempt to summarize some of the recent work, both fundamental and applied, on the rickettsiae. Much work on both phases has come from his own laboratory, and it is to be regretted that he has so little time to discuss so much.

Typhus fever is among the rickettsial diseases of major importance and is of concern not only in military preventive medicine, but also to civilian public health authorities. It is only natural that attempts to control this disease through immunization should be made, and a review of this field has fallen to Doctor Fox. Doctor Fox, taking advantage of the existence of an avirulent strain of *Rickettsia prowazekii*, has used this living organism as an immunizing agent. He will discuss the use of this "strain E" in volunteers and in large field trials and summarize present information on its efficacy.

PROBLEMS REVEALED BY THE EXPANDING USE OF TISSUE CULTURE PROCEDURES IN STUDIES ON INFECTIOUS AGENTS[1]

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Study of the viral pathogens of man has proceeded along a logical but unpredictable methodologic course. As the result of an enforced dependence on the living cell, the virologist has developed techniques whereby infectious inocula may be introduced into animals, old or young, or into cultures of their cells maintained in vitro. With each major advance in procedures for the manipulation of viruses has come a renewed burst of experimental activity. Thus, research in the past decade has been particularly influenced by the applications of viral hemagglutination, by the popularization of the suckling mouse as an agent for viral isolation, and by the use of tissue culture procedures.

The extensive reportorial coverage of the discovery of the Coxsackie viruses and of the in vitro propagation of the poliomyelitis viruses has tended to obscure contributions of earlier virologists working with similar technical approaches. Reference might be made to the team effort of the International Health Division of the Rockefeller Foundation on yellow fever, carried out in large part by members of this Society. Thus, Bugher, in 1941, enlarging on work by Theiler and Davis, demonstrated that baby mice were more susceptible than were older mice to yellow fever virus. The development of the 17D yellow fever vaccine remains the classic example of the practical application of in vitro methods.

In the study of infections of man, the in vitro procedure employing tissues of primates offers a convenient mechanism for viral isolation, for the propagation of such agents in quantity, and in many instances for the assay of specific antibody thereto—all without recourse to the experimental animal. We have reviewed aspects of the tissue culture field, in particular those related to problems of diagnosis and of etiologic investigation (Weller, 1954; 1955). Today we will be concerned with developments which indicate deficiencies in present knowledge. The selection of topics has been made to indicate problems, both fascinating and frustrating, that may influence effective utilization of the method. As a point of topical reference, let us consider the situation as it might affect the operations of a hypothetical team of virologists preparing to investigate febrile entities of unknown etiology in Uganda.

PROBLEMS

1. Problem posed by the "efficiency" of the technique. Paradoxically, the successes already achieved by use of the in vitro approach pose problems as regards its efficient utilization. It is apparent that for the isolation of viruses, in vitro techniques may equal or surpass in sensitivity more conventional methods.

[1] Current studies cited supported in part by research grant E-1023 from the National Microbiological Institute, National Institutes of Health, U. S. Public Health Service.

Influenza viruses, for example, may be isolated in tissue culture; Mogabgab (Mogabgab *et al.*, 1955) and Takemoto (Takemoto *et al.*, 1955) were, by this means, able to recover a greater number of influenza B strains than was possible in embryonated eggs. Comparable results have been reported with mumps virus (Henle and Deinhardt, 1955). Kjellén and Svedmyr (1954) prefer the human tissue culture system for the isolation of herpes simplex virus. These reports suggest that the hypothetical team of virologists must be prepared to differentiate the cytopathic changes induced by a variety of well defined agents, as well as those produced by recently isolated viruses responsible for entities such as varicella (Weller, 1953) and measles (Enders and Peebles, 1954).

Yet a more complex problem in differential diagnosis is posed by those cytopathic viruses falling into the general category of newly recognized agents. In 1951, we described the recovery of poliomyelitis viruses in vitro (Robbins *et al.*, 1951) and noted the isolation of other cytopathic viruses from human fecal material. One of the latter may be considered the first representative of an assortment of agents now variously referred to as "orphan viruses" (Melnick, 1954 A), the "human enteric" viruses (Ramos-Alvarez and Sabin, 1954), or simply "unidentified cytopathic agents" (Kjellén, 1955). The prevalence of these viruses is indicated by the experience of Horstmann (1955) in Egypt. Specimens were collected from 319 babies in Cairo suffering from mild illnesses; 113 were found to contain a cytopathogenic agent. Of the 113, 14 children were excreting poliomyelitis virus, 8 were infected with viruses pathogenic for mice, while from 91, agents of the "orphan virus" type were recovered. Equally striking is the study by Ramos-Alvarez and Sabin on healthy children in Ohio in which 25 agents were isolated. The heterogeneity of these new viruses is shown by the fact that while the majority of the Ohio agents belonged to three distinct serologic types, all were serologically unrelated to four antigenically distinct groups of "orphan viruses" previously defined in New Haven (Melnick, 1955).

Another new group of agents, lacking in pathogenicity for laboratory animals, is composed of the so-called adenoidal-pharyngeal-conjunctival or APC viruses described by Rowe, Huebner and co-workers in 1953. Similar or related viruses—termed the acute respiratory disease viruses (ARD)—have been recovered by Hilleman and Werner (1954); also by Neva and Enders (1954), and Kjellén (1955). At this Symposium, Dr. Huebner will acquaint us with the current status of these agents. It will suffice to emphasize the frequency with which representatives of this group may be isolated. This point is illustrated by the study at Fort Ord in which Dr. Lennette participated (Berge *et al.*, 1955); investigation of 201 cases of suspected influenza resulted in 43 isolations in vitro of ARD virus. Again it is desired to emphasize the immunologic heterogeneity of this group; Rowe and Huebner (Rowe *et al.*, 1955) recognize the existence of at least six immunologic types. There is evidence that differentiation as to type must rest on the performance of neutralization tests in vitro employing specific antisera. (Rowe *et al.*, 1955; Hilleman *et al.*, 1955).

Theoretically, these new viruses are separable from the 24 Coxsackie viruses (Dalldorf, 1955) on the basis of absence of pathogenicity for newborn mice. In

the laboratory such a differentiation is not always an easy matter. Melnick (1955) mentions an agent that required 11 tissue culture passages before it produced disease in infant mice on subinoculation. Conversely, serial passage in baby mice may be required—a tedious and expensive procedure.

In view of recent experiences with "new viruses", it would seem that the Uganda group might isolate a bewildering variety of agents and should be equipped with various viral antisera for the performance of neutralization tests. Yet these materials are not generally available. As an alternative then, the Uganda group might impose on fellow-workers for supplies of diagnostic sera or for assistance in identification. This time-honored procedure of mutual scientific assistance, best developed among friends or among groups operating under a common sponsorship, has been and will remain a useful mechanism. However, it imposes on the resources of the contributing scientist, and tends to isolate the individual researcher or group lacking in appropriate contacts. The rapid development of tissue culture procedures has magnified a pre-existing problem in virology. The hypothetical Uganda group, and virologists in general, would profit immeasurably if through the pooling of resources of commercial, philanthropic, and governmental agencies, standardized diagnostic reagents could be made available to all qualified workers.

II. Problems inherent in the tissue culture system. Establishment of a tissue culture operation per se in Uganda should not be expected to pose marked difficulties. Reliance could be placed on certified media (Porter, 1954) obtainable from commercial sources. A variety of tissues could be supplied by air. We have prepared cultures from tissue shipped from Sweden; Syverton and Scherer (1953) demonstrated that HeLa cell cultures would withstand shipment for long distances; and in New Haven, Melnick (Melnick *et al.*, 1955) has been using cultures made from trypsinized monkey kidney cells flown from India. However, the group might equally well rely on tissues, either of monkey or human origin, locally available.

Yet the question as to what tissue culture system or systems should be selected is a difficult one. Several considerations other than those of a purely technical nature would influence this decision, including: a) the susceptibility and sensitivity of the cell indicator system; and b) the frequency with which extraneous viruses, either latent or overt, might inadvertently be introduced with constituents of the system. Here it will be possible to mention only these aspects of a broad problem.

a) Cell susceptibility and sensitivity. A stable cell line manifesting a high degree of susceptibility for all human viruses and capable of being maintained in synthetic media would represent the virologists' idea of a utopian system. The potentialities of lines of human cells are being explored as, for example, the studies of Swim and Parker (1955) on fibroblasts, and of Chang (1954) on epithelial cells. The greatest amount of effort in this direction has been with the HeLa line, of which Dr. Syverton will shortly speak. The complexity of the problem is indicated by the fact that related viruses show marked variation in cytopathic potential for a particular cell type. For example, Crowell and Syverton (1955)

observed that only 4 of 16 antigenically distinct types of Coxsackie virus were cytopathic for HeLa cells. In a study confined to Group B strains of Coxsackie virus, Sickles and her co-workers (Sickles *et al.*, 1955) found that monkey kidney cells were more susceptible to destruction than was the outgrowth from human uterine tissue. These findings confirm earlier reports on the dissimilar cytopathic behavior of different Coxsackie viruses (Weller *et al.*, 1953; Stulberg *et al.*, 1954).

Variation in the relative sensitivity of cells is illustrated by studies dealing with the efficiency of the tissue culture method for the isolation of poliomyelitis virus. Available data now suggest the following decreasing order of sensitivity: human kidney cells, monkey kidney cells, HeLa cells, and monkey testicular cells (Kibrick, 1955; Neva, personal communication, 1955; Melnick, 1955; Wenner, 1955). These conclusions must remain tentative until techniques are further standardized. As another example, the extremes of susceptibility of some 13 strains of rat cells to Eastern equine encephalomyelitis virus as observed by Bang and Gey (1952) may be mentioned. The situation is complicated by the fact that variant lines of cells of altered susceptibility may spontaneously appear during cultivation; the existence of a substrain of HeLa cells that is relatively resistant to poliomyelitis virus is now established (Beasley *et al.*, 1955; Scherer, 1955).

Furthermore, in any consideration of this problem, it is improper to assume that the individual particles comprising a viral population are uniform as regards cytopathic potentiality. In the laboratory, it is possible to segregate genetic variants that differ widely in pathogenic characteristics as assayed in vivo and in vitro. Sabin's (1955A) studies have resulted in the segregation of a non-cytopathic strain of poliomyelitis virus.

These observations emphasize the difficulties now existent in the selection of a system of maximum sensitivity for the isolation of an agent. In one sense, no problem is posed if experiments are carried out with the realization that failure to isolate a cytopathic agent by in vitro means may reflect an insensitive system. With the rapid developments in methodology, we may conclude that this question as it now confronts the hypothetical Uganda virologists will be minimized with the passage of time—yet, in the absence of means to assay sensitivity of in vitro methods for the isolation of a virus, be it from a specimen or from a biological product, caution is indicated in interpretation of negative data obtained thereby.

b) *The problem of extraneous agents.* It is obvious that in the maintenance of a tissue culture system, and particularly if repetitive additions of biological products are involved, ample opportunity exists for the introduction of foreign viruses. These undesired agents may be introduced with the tissue or with constituents of the medium such as embryonic extracts or plasma. At present, the practical extent of the problem posed by "wild" agents thus derived is not known, but extraneous viruses are being encountered with increasing frequency. Gey (Gey *et al.*, 1954) has emphasized that so-called "non-specific" degeneration in a tissue culture may signify the presence of a viral contaminant, and has expressed the view that stable cell strains maintained in homologous media are in particular

jeopardy. In this connection, consideration must be given to the possible presence of latent viruses as well as to those that manifest themselves by overt cytopathogenicity. The virologist has learned to utilize colonies of experimental animals infected with latent viruses; a comparable situation appears to be developing in the tissue culture field.

Extensive use of monkey tissues in culture has revealed a variety of agents. With the exception of Newcastle virus as encountered by Chanock (1955), and traced to a lot of chicken plasma, the agents appear to have been derived from the monkey cells. In 1953, Rustigian (1955) employed cultures of monkey kidney for the attempted cultivation of dengue virus; uninoculated cultures developed vacuolated syncytial cell masses which were shown to be produced by a specific virus. Rustigian demonstrated his preparations to us, and in 1954 we noted the same type of viral contaminant (Enders and Peebles, 1954) (Weller, T. H., Cheatham, W. J., Bell, E. J., and Witton, H. M., 1955. Unpublished data). We have referred to Rustigian's agent as the monkey "foamy virus" in view of its cytopathic effect. This virus often is slow to appear in culture, and the fact that the changes are not unlike those produced by measles (Enders and Peebles, 1954) and mumps (Henle and Deinhardt, 1955) may cause confusion. A similar virus has been observed by us in uninoculated cultures of monkey testicular tissue; in this instance, the cytopathic changes were noted after periods of cultivation ranging from 26 to 55 days.

Other viruses have been recovered from cultures of monkey kidney tissues. B-virus has been reported by three different groups (Melnick, 1954B; Wood and Shimada, 1954; Krech and Lewis, 1954). Dr. Robert Hull has informed us personally that he has isolated eight new agents from monkey kidney cells; each produces a characteristic cytopathic effect and is immunologically distinct.

In the course of studies employing foreskin tissues, we have encountered two agents, apparently deriving from the culture system. The first is characterized by appearance of cytopathogenicity after a long latent period (30± days), tends to be focal, and produces a slowly progressive destructive process in fibroblasts in which collections of pycnotic nuclei persist as a prominent feature. (Weller, R. H., Cheatham, W. J., Bell, E. J., and Witton, H. M., 1955. Unpublished data.) This agent has been maintained for 6 tissue culture passages and is also cytopathic in monkey testicular cultures. The second agent, encountered more recently, (Neva and Weller, unpublished data), produces changes similar to those described for the APC viruses, but again appeared only after a period of cultivation of about 45 days. To date, we have not established the component of the culture system responsible for their introduction.

III. The problem posed by agents that manifest unusual in vitro behavior patterns. Lastly, we will mention the problem inherent in the peculiar in vitro behavior of certain viruses. The poliomyelitis viruses and similar agents have conditioned virologists to think in terms of agents that multiply rapidly, and are overtly cytopathic. As emphasis gradually changes, agents with quite different behavior patterns are being detected. Certain of the contaminating viruses previously

mentioned apparently persist for weeks in a latent phase before overt cytopathic changes become apparent.

An example of in vitro behavior that contrasts with that of the poliovirus group is afforded by the cultural pattern of varicella virus (Weller, 1953); subculture in this instance may be accomplished only by transfer of infectious cellular material. Spread of the agent in vitro, appears to take place primarily by passage from infected cell to susceptible contiguous cell. Black and Melnick (1955) have reported that the initial spread in vitro of B-virus occurs in a similar manner. At the moment additional agents that behave as does varicella are being defined. Dr. Margaret Smith has had under investigation for over a year a virus isolated from infants known to have salivary gland inclusion disease (Smith, personal communication, 1955). Last January we recovered a virus from liver biopsy material from a suspected case of cytomegalic inclusion disease. Shortly thereafter, Rowe (personal communication, 1955) sent us an agent derived from a culture of human adenoid tissue. These viruses, isolated independently in St. Louis, Boston and Bethesda, all initially in culture exhibit a slowly developing focal cytopathogenicity of a type resembling, but different from, that produced by varicella. They likewise produce intra-nuclear inclusions. On morphologic grounds they appear related. The discovery of these agents stresses the advisability of the routine utilization of cultures maintained for a minimum of one month following inoculation in any search for a new virus. Furthermore, consideration must be given to the utilization for subculture of inocula containing tissue as well as the customary supernatant fluids.

SUMMARY

In this paper we have outlined certain problems involved in the efficient utilization of tissue culture methods as applied to the study of the etiology and epidemiology of virus infections. In the particular sphere of interest of this audience —virus infections in the tropics—field investigations employing these new techniques are now beginning to materialize. That these will be profitable is a certainty—the reports of the propagation of dengue virus in the laboratory by Sabin (1955B), and by Hotta and Evans (1955), for instance, suggest an obvious subject for exploration in the field. In the planning of such field studies—in Uganda or elsewhere—an awareness of the problems discussed today might minimize the difficulties that certainly will arise. However, in the light of our limited knowledge at present, these problems cannot be entirely circumvented.

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CELLS IN CONTINUOUS CULTURE FOR STUDY OF VIRUSES[1]

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It is my privilege today to take part in this Symposium on Newer Knowledge of Viral and Rickettsial Diseases by reviewing with you the applications of cells in continuous culture to problems of concern to members of the American Society of Tropical Medicine. Let us consider these questions: What is meant by the term "stable cell strain in continuous culture"? What are the applications of this laboratory tool? How can cell strains be employed gainfully in experimental and epidemiological studies?

STABLE AND PURE LINE CELL STRAINS

Today human cells produced en masse by continuous culture are available for virus studies in laboratories around the world. Less than 30 years ago the possibility of such an achievement seemed fantastic; today fantastic possibilities have been reduced to laboratory problems concerned with convenience and simplicity. Tissue or cell culture was launched as a discipline in 1907. Doctor Ross Harrison, an experimental embryologist, combined the microsurgical procedures of Roux (1885) and Born (1896–1897) with the hanging drop technic familiar to bacteriologists to effect a unique experiment. By explant culture of frog nerve tissue in lymph on a coverslip, he was able to demonstrate the formation of a nerve fiber as an outgrowth of a single cell (Harrison, 1906, 1907). The new discipline approached maturity during the next 50 years through the notable efforts of Burrows, Carrel, the Lewises, Fischer, Parker, Gey, Earle and others, who explored the nutritional and environmental conditions needed for continued propagation and maintenance of cells. This evolution has resulted in the acceptance of in vitro culture as a means for supplying biologically active mammalian cells freed from the complex environment of the parent organism. The natural desire to observe cultivated cells for long periods of time led to the development of *stable cell strains in continuous culture*. Primary cell cultures commonly were derived by explanation, usually in a plasma clot, of small fragments of biopsied tissue minced with sharp knives by scissor-like action. From such primary cultures, cell colonies can be propagated serially by subdivision of outgrowth at each generation. Eventually through competition a predominant cell type emerges in the population to give rise to a single morphologic strain. During successive serial transfers in a constant environment the cell strain assumes a stable morphology, metabolic activity and rate of multiplication.

A more critically defined cell population in continuous culture, the *pure line cell strain*, is derived from clonal propagation from a single isolated cell. The best known example is the L strain of Earle, established by Sanford, Earle and

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Likely (1948). The strain was derived from a single cell of a line of fibrocytes, originally established from the subcutaneous tissue of a normal C3H mouse, which had acquired malignancy after in vitro treatment with 20-methylcholanthrene. Strain L cells have been employed extensively by Earle and associates in studies of cellular nutrition. More recently Puck and Marcus (1955) have described additional technics for the isolation of pure cell strains.

The origin of cell culture was soon followed, although intermittently, by application of cultivated cells to the propagation of viruses. A shift in emphasis from the plasma clot culture resulted from the introduction by Maitland and Maitland (1928) of the suspended tissue fragment technic. The new procedure was received with such enthusiasm that most viruses, perhaps all, were tested for their capacity to propagate in the suspended cell culture. Many viruses were so propagated. The Maitland technic was and still is valuable for its intended purpose, virus production. The substitution of tissue fragments for cultivated cells of known constitution, however, slowed the progress of virus biology. Because some viruses could not be propagated in suspended fragments of the host tissue indicated by in vivo trophism, it was assumed that these agents could not be propagated in vitro. It remained for Enders, Weller and Robbins (1949) to demonstrate extraneural propagation of polio viruses. The classic remark of Louis Pasteur, "In the field of observation, chance favors only the prepared mind", applies cogently to the success of Doctor Enders and his associates with in vitro cultivation of polio virus and their recognition of cytopathogenicity and lack of pH change as practical indicators of virus activity. These indicators had received only limited notice after their description by earlier investigators.

The development of cell culture has been greatly accelerated in the past decade by the replacement of art with science. It must be admitted that art has not entirely been replaced. The application of bacteriologic asepsis with help from antibiotics has made possible the practice of cell culture in the average laboratory. Three basic methods are employed now for the cultivation of cells in vitro. 1) Cells or tissue fragments are placed directly on a glass surface to propagate as a cellular monolayer. 2) For larger populations the available substrate surface is increased by the use of glass cloth, glass wool, glass helices, or of gelatin or cellulose sponge. 3) Dispersed cells are propagated in suspension in a liquid medium. Although the latter method promises great advantages, it is still under development and will not therefore be considered further here. The most important single requirement for a successful continuing program of cell culture is common sense. Cell culture, even on a large scale, can be performed satisfactorily with standard equipment available from commercial sources when simple adequate procedures are employed. The roller drum and the Carrel flask, however useful for specialized purposes, are not essential to the use of mammalian cells in vitro for the study of infectious agents.

Applications of cells in continuous culture to the study of viruses may be placed in two categories: 1) Investigational, for the exploration of a) the nature of cellular trophism, b) the formation and structure of viral inclusion bodies,

c) the comparative metabolism of infected and uninfected cells, d) the effect of antimetabolites upon infection, e) the mode of antibody action upon viruses, and f) the mechanism of viral interference. 2) Practical for a) isolation, typing and assay of virus, b) assay of neutralizing antibody and c) production of viral antigens and vaccines. How the cells are prepared for these theoretical and practical purposes and with what gain, is best shown by example. I shall cite, with your indulgence, the studies most familiar to me: those carried out in collaboration with staff members and graduate students of the Department of Bacteriology of the University of Minnesota.

Since 1949 major attention has been given in our laboratory to the development of methods for the large scale production of cells in continuous culture. It was recognized that a supply of cells in quantity would permit the study of host-virus relationships at the cellular level. Of particular interest was the possibility that such cells would satisfy the urgent need for an accurate, rapid method for isolation of poliomyelitis virus and assay of antibody. The development was aided by a personal background of intermittent experience with tissue culture accumulated since 1934, by encouragement from the significant achievements of Enders, Weller and Robbins (1949, 1955) and by discouragement over the continuing problem of obtaining fresh biopsy material from patients or monkeys. The efforts so stimulated resulted in quantity production of cells from five stable human strains, one mouse strain and one monkey strain. The seven strains included three epithelial and four fibrocytic. Each has been used for rapid and precise indication of the infectivity of a known spectrum of viruses, and of the inhibitory effect of corresponding specific antibodies. Tests are simple in principle and performance. Cells of six of the seven stable lines undergo total destruction after exposure to poliovirus or to many other viruses infectious for man; only the L strain of Earle is not susceptible to wild strains of poliomyelitis virus. The destructive cytopathic response facilitates recognition of viral activity. Antibody is detected in the serum by addition to known virus in suspension before transfer to cell cultures, as for neutralization test, or by addition to cell cultures before exposure to virus, as for protection test. If the serum contains homotypic antibody the cells are protected; if not, they are destroyed. Conversely, inhibition of cytopathic effect by known homotypic antibody serves to identify unknown virus.

The HeLa strain of human malignant epithelial cell is most representative of the stable lines in continuous culture since it has been employed more extensively than any other for viral and antibody assays. Both it and Earle's pure line L strain are readily available to competent investigators. Since HeLa has satisfied the need for a highly susceptible human cell strain capable of yielding poliovirus and other viruses in quantity in from one to several days, in our laboratory it has replaced the less convenient explants of human or monkey tissue, and the far more expensive intact monkey.

On February 8, 1951, Dr. George Gey explanted biopsy tissue from an epidermoid carcinoma of the human cervix, and maintained cultures by serial transfer (Gey *et al.*, 1952). After 30 generations passed up to May 31, 1952, cells

in plasma coagula were sent to the University of Minnesota. Stock cultures of the explants were established, weaned from the plasma substrate, moved from roller tubes to larger bottles, and finally propagated in monolayer after intercurrent dispersal with trypsin at each passage (Scherer *et al.*, 1953). The developed capacity for preparation in cellular suspension made possible routine production of cultures in large numbers.

Once the ready applicability of strain HeLa to isolation of virus and assay of antibody was established, and availability of cells in quantity had been achieved, one could foresee extensive application to many experimental and practical problems in epidemiology and public health. It was important to learn the range of susceptibility and cytopathologic response of these cells to viral infection. For example, strains representative of 16 antigenically distinct types of C virus were selected for study to learn the effect of each virus upon cells of strain HeLa. Of 16 immunologic types of C virus, 4 types proved cytopathogenic for strain HeLa cells resulting in from 1 to 4 days in total cellular destruction and multiplication of virus. Cytopathological effects were observed for 3 strains of Dalldorf's B-3 (inclusive of Melnick's 'Nancy'), Conn.-5 (Dalldorf's, B-1), Melnick's Texas-13 (B-4), and Texas-14. The destructive process was indistinguishable from that of poliomyelitis infection. Unequivocal multiplication of each cytopathogenic type was demonstrated by growth curves and serial passages where infectivity persisted despite cumulative dilution beyond the extinction level of the inoculum. The cytopathologic activity of each virus was neutralized in cell culture tests by the homotypic anti-Coxsackie monkey serum; it was not neutralized by antisera to the three types of poliomyelitis virus or to heterotypic C viruses. Similar studies (Scherer *et al.*, 1953; Syverton *et al.*, 1954; Syverton and Scherer, 1954; Scherer and Syverton, 1954; Scherer and Syverton, 1954; Scherer and Syverton, 1955; Crowell and Syverton, 1955) showed that the viral range of HeLa strain cells included the viruses listed in Table 1. Probably other as yet untested viruses will be added to the list in time. Passage in strain HeLa resulted in multiplication of each virus and in destruction of cells by each of the viruses listed with the exception of two, St. Louis encephalitis and Japanese B encephalitis (Scherer and Syverton, 1954). For these two viruses, cytopathological effects were observed in the first or in several early passages; in successive passages the supernatant fluids yielded virus without overt evidence of cellular destruction. Comparative studies (Scherer and Syverton, 1954a, 1954b, 1955) have been made of the cytologic changes resulting from infection of strain HeLa by the viruses of herpes simplex, pseudorabies, vaccinia, Eastern equine encephalomyelitis, Western equine encephalomyelitis, West Nile, and pseudolymphocytic choriomeningitis, and by the three types of poliomyelitis virus.

In comparison with the viral range of HeLa, that of Earle's L strain includes herpes simplex (Scherer, 1953); pseudorabies (Scherer, 1953); encephalomyocarditis, lymphocytic choriomeningitis and pseudolymphocytic choriomeningitis (Scherer, 1953); and Western equine encephalomyelitis (Chambers and Evans, 1953). Viruses not propagated were polio virus, mouse encephalomyelitis, GD-7 strain (unpublished experiments) and Rous sarcoma virus (Sanford *et al.*, 1952).

TABLE 1

*Viruses propagated in cultures of human epithelial cells, strain HeLa**

Poliomyelitis group†	*Arthropod-borne encephalitogenic group*‡
Poliomyelitis, Type 1	Western equine encephalitis
Poliomyelitis, Type 2	Eastern equine encephalitis
Poliomyelitis, Type 3	West Nile
Coxsackie group§	St. Louis encephalitis
Coxsackie, B-1	Japanese-B encephalitis
Coxsackie, B-3	*Epitheliotrophic group*¶
Coxsackie, B-4	Vaccinia
Coxsackie, Texas-14	Herpes simplex
Miscellaneous group‖	Pseudorabies
Pseudolymphocytic choriomeningitis	

* Multiplication and cytologic changes for all viruses except viruses of St. Louis and Japanese-B which were not cytopathogenic.

† Syverton, *et al.*, 1953, 1954; Syverton and Scherer, 1954.

‡ Scherer and Syverton, 1954.

§ Crowell and Syverton, 1955.

¶ Scherer and Syverton, 1955.

‖ Scherer and Syverton, 1955.

This brief summary of the susceptibilities of only two stable cell strains in continuous culture demonstrates the power and versatility of these new tools. It is not too much to expect that the list of agents susceptible to study will expand rapidly. A broad spectrum of cell types in continuous culture is being developed for the study of viruses through the continuing efforts of many workers. Notable for their contributions are the laboratories of Gey and Bang, Parker and Swim, Stulberg, Snyder and Chang, Lahelle and others in this country and abroad.

TABLE 2

Distribution of isolates by immunotype from 922 patients

Specimen from	Total by Year	Immunologic Type		
		T1	T2	T3
1946	7	7	0	0
1948	59	59	0	0
1949	20	9	11	0
1950	54	21	1	32
1951	15	12	2	1
1952	85	77	0	8
1953	394	385	1	8
1954	182	53	43	86
1955	106	63	22	21
Total	922	686	80	156
Distribution by type		74%	9%	17%

The value of cells established in continuous cultures for epidemiological studies is exemplified by some results obtained by employment of HeLa cells for the rapid laboratory diagnosis of acute poliomyelitis and for the specific allocation of field strains by immunologic type. Strains of poliomyelitis virus were isolated from 922 patients, from Minnesota and neighboring states, admitted with a clinical diagnosis of poliomyelitis to hospitals in Minneapolis from 1946–1955. The specimens for study included feces, throat washings and brain or cord tissue. Viruses were isolated in from 1 to 6 days, commonly in less than 48 hours. Isolation and typing can be accomplished in a single step. The results are presented in Table 2.

PRODUCTION OF STRAIN HeLa CELLS

The procedures used in the studies cited are not difficult, and can be applied with suitable modification to other strains in continuous culture. Since detailed protocols for the production of strain HeLa cells and performance of assays have been published with schematic directions (Scherer and Syverton, 1954), only a brief description of the general method with comments on recent modifications will be given here. For production purposes, sheets of cells propagated on the glass walls of bottles are dispersed with trypsin, resuspended in a nutritive medium of 40 parts human adult serum and 60 parts Hanks' balanced salt solution, and enumerated in a hemocytometer. After dilution aliquots of the cellular suspension are transferred to other bottles for further production of cells, or to tubes for use in virus or antibody assays. A mechanically driven helical stirring rod keeps the cells in suspension for delivery of the 0.5 ml. aliquots by a Cornwall pipetting unit. Each bottle culture yields up to 200 tubes, which are incubated in a stationary position inclined at an angle of 5°. In this manner a comparatively simple procedure provides bottles of cells for production and tubes for immediate use. The methods developed experimentally for such massive production of cultivated mammalian cells have been adopted by two commercial firms so that quantities of cells are available to any laboratory by order.

As might be expected, the suitable preparation of contaminated specimens is an important prerequisite to successful virus isolation. The treatment recommended as routine, which relies upon differential centrifugation and use of antibiotics, to combat contamination, can be applied to material containing a mixed microbial flora. With sterile specimens and clear supernates of body fluids or secretions, treatment often can be reduced or omitted. The preparation of grossly contaminated specimens for virus isolation is best illustrated by the treatment given feces—a fecal specimen obtained in a paper carton from a hospitalized patient contains about as widely variable a microbial flora as will be encountered. The protocol is as follows:

1. Place 6 gm. of feces in a 60 ml. (32 x 125 mm.) sterile tube containing 30 ml. sterile glass-distilled or deionized water, with 2.0 mg. per cent of phenol red.
2. Emulsify the specimen with a sterile tongue depressor, or shake horizontally at 450 oscillations per minute for 30 minutes at 4°C.

3. Sediment for 30 minutes at 18,000 r.p.m.
4. Transfer the supernatant fluid to a second sterile tube, and add per ml. of supernate 500 units of penicillin and 300 μg. of streptomycin contained in 0.05 ml.
5. Store the treated specimen at 4°C. for 1 hour and/or at −20 to −70°C. until tested.

Cells dispensed into tubes and propagated in monolayer must be prepared further for use in virus studies. Removal of viral inhibitor(s), such as antibody contained in the serum employed for cultivation, is effected by washing the cells with balanced salt solution until the original fluid overlying the cells is diluted at least 1:10,000. Three 1 ml. washes for each tube suffice.

As has been indicated, the interpretation of tests for virus or antibody activity is facilitated by observation of a fulminant cellular degeneration, which usually appears in from 1 to 6 days after injection and progresses rapidly to total destruction over a period of 12 to 24 hours. This cytopathic effect on HeLa cells is produced by the viruses listed in Table 1 (Scherer *et al.*, 1953; Syverton *et al.*, 1954; Syverton and Scherer, 1954; Scherer and Syverton, 1954; Scherer and Syverton, 1954; Scherer and Syverton, 1955; Crowell and Syverton, 1955), by viruses of the APC-ARD groups (Huebner *et al.*, 1954; Ginsberg *et al.*, 1955), and by still other viruses tested by other investigators.

EMPLOYMENT OF CELL STRAINS

Commonly *isolation of an unknown virus and immunologic typing* is accomplished at the same time. Known homotypic antibody is essential to specify the identity of unknown virus, and to establish that any cytologic changes have been produced by virus infection and not by indeterminant "toxic" material in the test inoculum. The method employed for the immunologic identification of unknown field strains of cytopathogenic poliovirus is representative of the general technic. For each test, 8 tubes containing from 60,000 to 100,000 HeLa cells from 4 to 7 days old are employed. The 8 tubes are washed to remove any antiviral substance and labeled for identification of unknown material or of patient by name and number. Normal serum is added to the first 2 tubes, anti-type 1 serum to the second pair, anti-type 2 to the third pair and anti-type 3 to the fourth pair. An aliquot, 0.1 ml. of the specimen under test for viruses is added to each tube. The tubes are observed microscopically daily. The presence of polio virus is revealed in from 1 to 6 days by destruction of cells in 6 of the 8 tubes. The unaltered cells in two tubes reflect the protective or neutralizing effect of the homotypic antibody and thereby reveal the immunotype of the isolated virus as Type 1, 2 or 3. This procedure is in essence a protection test since antibody is added to the cells before virus.

Quantitative assays of virus or antibody can be performed with equal ease (Syverton and Scherer, 1954) The schematic drawing shown in Figure 1 illustrates the methodology for virus assay and the results of a test. The first set of tubes contains diluent to effect dilutions of the test virus suspension within a known range; the second set represents the five tube cultures of strain HeLa

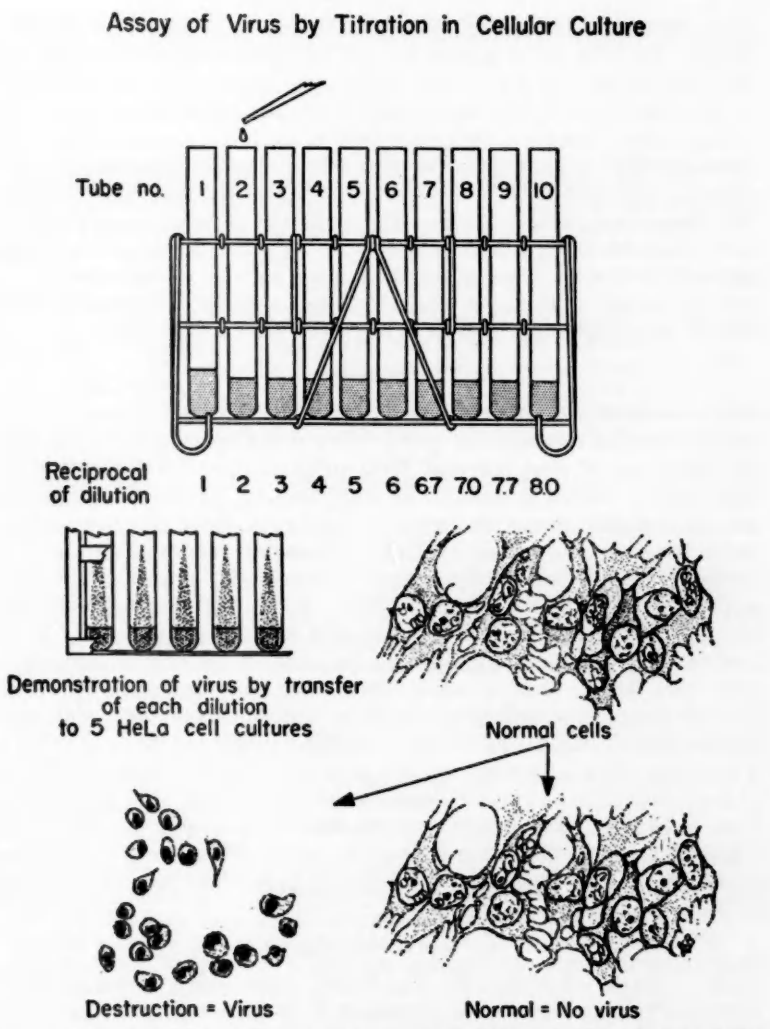
Assay of Virus by Titration in Cellular Culture
Tube no. 1 2 3 4 5 6 7 8 9 10
Reciprocal of dilution 1 2 3 4 5 6 6.7 7.0 7.7 8.0
Demonstration of virus by transfer of each dilution to 5 HeLa cell cultures
Normal cells
Destruction = Virus
Normal = No virus

FIG. 1. Assay of virus by titration in cellular culture. Schematic drawing to illustrate the methodology for virus assay and the results of a test. The first set of tubes contains diluent to effect dilutions of the test virus suspension within a known range; the second set of five cellular tube cultures of strain HeLa are for detection of virus; the normal HeLa cells upon infection by virus are destroyed (lower left), or remain unchanged (lower right) in the absence of virus.

employed for detection of cytopathogenic virus in each dilution. Five tubes per dilution are used for statistical reasons. The results of the test are readily obtained; normal HeLa cells upon infection by virus are destroyed (lower left) or in the absence of virus remain unchanged (lower right). An essentially similar protocol can be employed for quantitation of antibody, or other virus inhibitor, antimetabolite or antibiotic. The first set of tubes with known amounts of diluent permits dilutions of the test serum within a known range for mixture with a constant amount of virus. The second set of tubes is employed to test each successive decrement of antiserum for its ability to neutralize the pathogenic effect of virus. If unneutralized virus is present, the cells are destroyed. The persistence of unaltered cells, which demonstrates protection by antibody against the cytopathogenic effect of free virus, reveals the endpoint.

SUMMARY

The availability to qualified laboratories of the stable cell strain in continuous culture satisfies the need by physicians and epidemiologists for a practical laboratory test of viral infection. With aid of this test the diagnosis of illness suspected on clinical grounds to be mild, atypical, or abortive poliomyelitis, aseptic meningitis, or encephalomyelitis, may be completed. The morphologically stable human cancer cell strain, HeLa, reveals the activity of a wide range of viruses by total destruction after infection. Conversely, antibody in the patient's serum is detected by its capacity to block the cytopathic action of homotypic virus. This inexpensive, rapid and accurate diagnostic test can and has been used in many laboratories, particularly for isolation and immunologic typing of polio virus. Throat washings, stool samples, and blood or tissue specimens from patients suspected to have poliomyelitis or other disease of the central nervous system provide material for study. Commonly, results can be obtained in from 1 to 4 days. HeLa cells have been used at the University of Minnesota for study of more than 3500 patients. Wild strains of poliomyelitis virus were recovered from more than 900 patients, and identified by immunotype. The methods described here may be used or adapted by qualified workers to epidemiological studies or other investigations of the most intimate host-parasite relationships at the cellular level.

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IMMUNIZATION AGAINST POLIOMYELITIS WITH LIVING ATTENUATED VIRUS[1]

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"No man can answer for his courage who has never been in danger."

LaRoche

The immunization of man against poliomyelitis with preparations of living attenuated virus has been practiced by myself and my associates for the past five and one-half years (Koprowski *et al.*, 1952). All the results of the study cannot even be summarized in this lecture and, while I shall review some past events, I intend to concentrate on taking an inventory of the present.

Strains of virus. Three attenuated strains of virus have been used for the immunization of human subjects: two, the TN strain (Koprowski *et al.*, 1952) and MEF_1 strain (Roca-Garcia *et al.*, 1952; Roca-Garcia and Jervis, 1955) represent Type II, and one, the SM strain (Koprowski *et al.*, 1954a), represents Type I. The TN strain is a rodent-adapted virus. It was administered for immunization purposes after eight mouse brain passages or, more recently, after 27 to 30 passages in the central nervous system of PRI mice. The MEF_1 strain was used after 70 passages in developing chick embryos. The type I SM strain is also rodent-adapted, and can be maintained in chick embryo tissue culture for several generations. The latter material was used for feeding. All three strains are nonpathogenic for intracerebrally injected monkeys, but occasionally a monkey injected intraspinally with a high concentration of virus will be paralyzed. Besides serological distinction, the three strains differ in their cytopathogenic properties (Koprowski, 1955a). The Type II TN and MEF_1 strains (Roca-Garcia and Jervis, 1955) are noncytopathogenic for tissue culture-grown fibroblasts or epithelium of either simian or human (normal and neoplastic) origin. The Type I SM strain is cytopathogenic. I shall return to these properties later when discussing the duration of alimentary infection in relation to cytopathogenicity.

"Let not the remembrance of thy former trials discourage thee."

Thomas à Kempis

Clinical trials. The organization of the clinical trials followed a certain established pattern (Koprowski, 1955b). Individuals, mostly children, were bled, and

[1] This paper presents, in abbreviated form, results of a large-scale clinical trial, a full description of which will be found in a paper entitled "Clinical Investigations on the use of Attenuated Strains of Poliomyelitis Virus as a Method of Immunization of Children with Living Virus" by H. Koprowski, T. W. Norton, G. A. Jervis, T. L. Nelson, D. L. Chadwick, D. J. Nelsen and K. F. Meyer, *J.A.M.A.* **160**: 954–966, 1956.

the antibodies in their blood determined by tissue culture neutralization tests. Only subjects lacking antibodies to the type of virus to be administered were included in a trial. In some situations, an attempt was made to secure subjects who had no antibodies against any of the three types of virus.

Virus was always given by the oral route, either in milk or in a hard gelatin capsule (Type I only) (Koprowski, 1955b). All subjects fed virus were quarantined and subjected to rigid clinical observation. Attempts to isolate virus were made on stool specimens collected daily (and then pooled), and occasionally on blood samples. The development of antibodies was followed in serum samples drawn at regular time intervals after the administration of virus.

One hundred and fifty subjects have received the TN (Type II) strain, 75 the SM (Type I), and 18 the MEF_1 (Type II). The results for the latter group are still in the preliminary stages of evaluation, and will not be discussed at present.

> "Omit the negative propositions. Nerve us with incessant affirmatives."
>
> Emerson

Negative findings. None of the 243 subjects, the majority of whom were children, showed signs of sickness which could be attributed to the ingestion of virus. The spinal cords of three subjects who died of incurable diseases one to two years after the administration of the attenuated viruses were found to be free from histologic lesions of poliomyelitis (Jervis, 1955). No virus has as yet been isolated from the blood of individuals who received either Type I or Type II virus (Koprowski, 1955c). Eight subjects who drank a "milk shake" containing Type I (from $10^{2.8}$ to $10^{4.9}$ infectious tissue culture doses) and Type II ($10^{5.0}$ mouse paralytic doses) had their throats swabbed daily. The swabs were examined for the presence of virus, and none was ever found. Using either mice or tissue culture as medium, the noncytopathogenic Type II strains have been isolated from fecal material only on rare occasions, and then in concentrations never exceeding $10^{1.0}$ PD_{50} (mouse paralytic doses) per gram of fecal material. In contrast, the cytopathogenic Type I SM strain has been isolated from the stools of every subject fed the virus (Koprowski, 1955b).

> "Nothing can satisfy, but what confounds.
> Nothing, but what astonishes, is true."
>
> Young

Susceptibility of the human alimentary tract to infection with attenuated strains. Evidence accumulated in the course of clinical trials and paralleled by laboratory investigations has indicated that man is the most susceptible species to poliomyelitis infection—as compared, for instance, with chimpanzees or monkeys (Koprowski *et al.*, 1954b). The question arose: how small a concentration of virus will infect man? Recently completed trials furnished an opportunity to examine this problem. Groups of nonimmune children were fed either the SM strain in capsules or the TN strain in milk. The amount of SM virus administered in serial falling dilutions was determined, as shown in Table 1, by "plating out" on a monolayer

TABLE 1

Relationship between dosage and alimentary infection—Type I virus

PFP of Virus Fed	Subject* No.	Duration of Alimentary Infection (days)	Antibody Titer†
20	44	4–21	1.8
	45	8–35	2.4
	46	3–44	1.8
	47	3–75	2.4
2	48	1–44	1.8
	49	3–30	2.4
	50	None	0
0.2	51	None	0
	52	None	0

PFP = Plaque forming particles of virus.

* Subjects had no antibodies to Type I or Type II prior to feeding. All received the virus in capsule form.

† In logs to the base of 10.

of monkey kidney epithelium, and counting the number of plaques produced (Dulbecco and Vogt, 1954). The results, measured by the rate of alimentary infection and by antibody response, seem to indicate the susceptibility of man to a dose as small as two plaque forming particles of Type I virus. Whether this is a true experimental finding depends on several factors which remain to be clarified. The diluent used for the preparation of the virus capsule may have interfered in some way with the determination of virus concentration in tissue culture. It would also be important to count the number of plaques of virus grown on a monolayer of epithelium of human (Kibrick, 1955) and not of simian origin. Noteworthy, too, is the fact that the virus administered by capsule was, in a way, placed in immediate contact with a particularly susceptible segment of the intestinal tract—possibly some lymphoid tissue such as Peyer's patches (Bodian, 1955).

Results of a similar trial in which the Type II TN virus was used are shown in Table 2. The laboratory determination of the infectivity of the inoculum had to be done in mice because of the noncytopathogenic character of the virus strain. This method is much less exact than that employed for Type I virus. Again, however, man has been found susceptible to a dose as small as 300 PD_{50} of virus, although one, or possibly two, subjects escaped infection with a tenfold greater amount of virus. Antibody response was used as the criterion of infection since, as was mentioned before, excretion of this virus occurs sporadically and in low concentration. The Type II virus, in contrast to the Type I, was not placed in direct contact with the gastric and intestinal mucosa, and therefore the data are actually not directly comparable. Since Type II virus was administered in liquid form, it may have multiplied first in the throat, with a secondary multiplication in the gut. This is very unlikely, because of subsequent negative findings on

TABLE 2

Relationship between dosage and alimentary infection—Type II virus

Mouse PD_{50} of Virus Fed*	Subject No.	Duration of Alimentary Infection (days)	Antibody Titer*
4.5	34	None	2.4
	35	12–14	2.8
	36	11–16; 21–22	2.4
3.5	37	None	0
	38	None	2.4
	39	None	0.6
2.5	40	12–14; 18–22	2.8
	41	None	1.2
1.5	42	None	0
	43	None	0

* Expressed as logs to the base of 10.

virus isolation from the throat region. Conversely, the "travel" of Type II virus from the mouth to the intestinal mucosa may not be as smooth an operation as direct contact of a capsule with the stomach or duodenum; obstacles to viral penetration and multiplication may account for the lesser susceptibility of man to Type II infection as compared with Type I. However, the simplest and most plausible theory is that the two strains, representing two types of virus, possess different properties, serological and otherwise.

"When rich villains have need of poor ones,
poor ones may make what price they will."

Shakespeare: *Much Ado About Nothing*

Attenuated virus as a contagium. Today the main objection of public health authorities to the more widespread use of live attenuated viruses in clinical trials is the fear of contagion. Without looking into the relative merits—and they are very relative indeed—of this objection, the problem demands a factual answer.

There are formidable odds against the contagious nature of the TN strain because of the character of the virus. Evidence accumulated from the successive trials conducted during the past 5 years (Koprowski *et al.*, 1952; Koprowski, 1955c) has indicated that the Type II virus is not contagious: i.e., cannot be passed from one subject to another. However, the cytopathogenic SM Type I virus, which is excreted from the human intestinal tract more regularly and at higher concentrations (Koprowski, 1955b) poses another problem. Unless investigated, this may be caviled at *ad nauseam*.

Six children who had no antibodies against Type I and II were fed simultaneously the SM and TN strains in concentrations stated in Table 3. Type I was administered in capsule form; Type II in milk. None of the six was found to excrete Type II virus, but they all became intestinal carriers of Type I virus,

TABLE 3

Transmission of attenuated poliomyelitis virus from man to man through contact

Groups and No. of Subjects	No Pre-Feeding Antibody to Types:	Type Fed	Intestinal Carriage‡	
			Type I	Duration in days
Donors: 6*	I, II	I, II§	6/6	1–66 1–83 4–65 5–16 6–9 16–26
Contacts: 8†	I, II, III	None	3/8	4–66 10–85 11–98

* Fed virus.
† Contacts of those fed.
‡ No intestinal carriage of Type II.
§ 200 PFP of SM strain and 90,000 mouse PD_{50} of TN strain administered.

and all developed homotypic antibodies two weeks after the administration of virus. These six subjects were kept in close contact with eight other children who had no antibodies against any of the three types of poliomyelitis virus. Starting from the day of virus administration, for three hours a day for 20 days, the six "donors" and the eight contacts were kept together on a large mat, the cover of which was not disinfected or changed at any time. These children moved about, played together and used common toys. None of the donors wore any clothing over their diapers, but these were changed promptly if subjects defecated during the "playtime."

Every single stool specimen of the eight contacts was processed for virus isolation, and blood specimens were tested for the presence of Type I antibodies. The results (Table 3) indicate that three of the eight contacts became infected with Type I virus. In one case, the contact infection must have occurred immediately, since virus was found in her stool on the fourth day after the six donors were fed. The other two contacts who became infected did so later, as indicated by the first appearance of fecal virus on the 10th and 11th days, respectively. None of the three recipients showed any signs of illness, and all developed Type I antibodies on the 27th day after being placed in contact with the six donors. The remaining five contacts were kept under constant surveillance for 16 days following their separation from the donor group. They did not excrete virus and had no antibodies. Following this period, they were fed Type I and II viruses and promptly became intestinal carriers and developed antibodies.

It may be of interest to mention that in contact with the six children comprising the donor group were nine nurses, who provided constant nursing care for their little patients. Seven of the nurses had no antibodies against Type I at the start of the clinical trial. Two and one-half months later, they still had no anti-

bodies against Type I. One may deduce from these observations that the attenuated Type I virus is not very contagious. The three to eight ratio of infected contacts, supplemented by the results observed in the nurses, supports this averment.

A question may be raised relative to the correlation of these results with the fact that the human intestinal mucosa seemed to become infected with two plaque forming particles of the same SM strain. Two hypotheses have to be investigated: the less probable is that the amount of virus made available to the contacts by the donors was very small indeed—less than two plaque forming particles. Because of the intimate nature of the contact, this does not seem likely. The second hypothesis would postulate the existence of formidable obstacles encountered by the virus in its attempt to travel from the nose or mouth down to the intestinal tract, barriers detoured by placing the two PFP of virus in immediate contact with the intestinal mucosa.

Since the low degree of contagion applies at present only to an attenuated—through laboratory manipulation—form of virus, generalization on the basis of the findings may be inadvisable. It is quite clear, however, that when the principles of simple personal hygiene are practiced, the attenuated SM virus may not pass from one subject to another. Eminent authorities on the danger of contagion will have less to cavil at and more to think about—a state of affairs not at all unsalutary.

Protectio trahit subjectionem et subjectio protectionem.

Latin law

Virus and immune globulin: simultaneous administration. Although the results of mass administration of immune serum globulin to human subjects during the

TABLE 4

Effect of immune serum globulin upon the carrier rate in subjects fed Type I and Type II simultaneously

Group Receiving	Subj. No.	No Pre-Feeding Antibody to Virus Types	Duration of Carrier State—Type I† (days)	Antibody Response	
				Type I	Type II
Immune globulin	5	I, II, III	1–23; 29–58; 64–69	+	+
	7	I, II, III	4–111	+	+
	17	I, II, III	None	−	+
	21	I, II, III	2–38; 46–52	+	−
	28*	I, III	3–12; 16–20	+	
	52*	I	1–28; 39–41; 53–63	+	
No immune globulin	13	I, II, III	3–58; 64–69	+	+
	14	I, II, III	11, 23–38; 49–52	+	−
	23	I, II, III	1–52	+	+
	27	I, II, III	1–90	+	+
	25	I, II, III	None	−	+

* Received Type I only.

† No Type II virus was excreted.

epidemic of 1953 were not very encouraging (Hammon, 1954), its protective value against paralytic infection in monkeys had been ascertained by Bodian (1952). It was of interest to determine the effect of immune globulin injection upon the simultaneous feeding of attenuated viruses. Six subjects (Table 4) received one inoculation of immune serum globulin intramuscularly, 0.15 ml. per pound of body weight At the same time, all six received 200 plaque forming particles of Type I virus, and four of the six were also fed $10^{4.6}$ PD_{50} of Type II. Five other subjects (see Table 4) received the two viruses but no immune globulin. The results of the trial seem to indicate that the administration of immune serum globulin has no effect whatsoever on the duration of the intestinal carrier state nor on antibody development following the feeding of live viruses. None of the eleven subjects was found to excrete Type II virus, and two—one in each group—failed to excrete Type I. This can be attributed to interference between Types II and I, and obviously had no relation to the use of immune serum globulin.

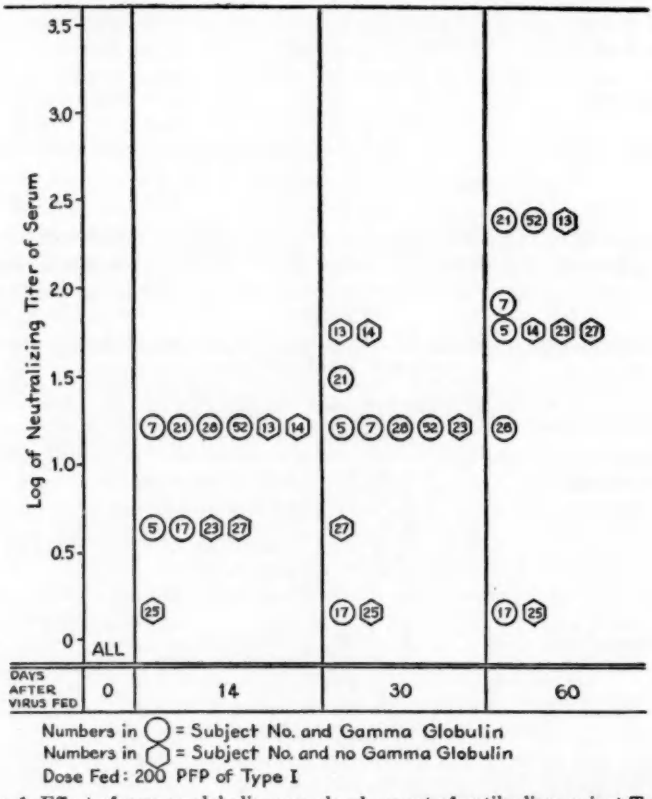

FIG. 1. Effect of gamma globulin upon development of antibodies against Type I.

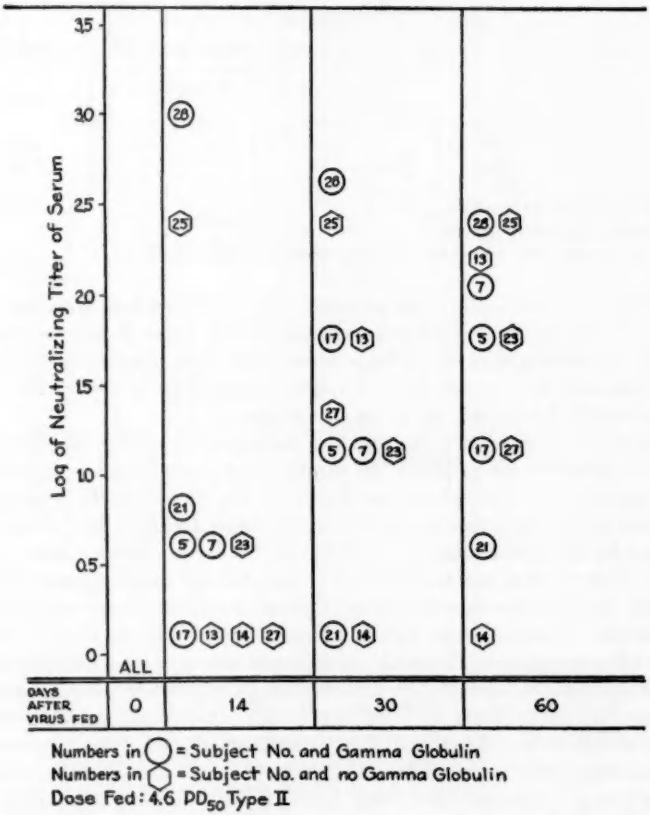

FIG. 2. Effect of gamma globulin upon development of antibodies against Type II.

The lack of influence of immune serum globulin upon the actual level of antibodies developing as the result of virus administration is illustrated in two figures: Fig. 1 showing Type I antibodies and Fig. 2, Type II antibodies. Once more, it was practically impossible to distinguish between subjects who received globulin and those who did not. The failure of two individuals to develop Type II antibodies may again be ascribed to interference.

> "If an idiot were to tell you the same story every day for a year, you would end by believing him."
>
> Burke

Repeated administration of virus—carrier rate and antibody response. The results of re-exposure to a viral homotype of a person who already has antibodies remain controversial, principally because of a dearth of quantitative observations.

TABLE 5

Effect of re-feeding‡ Type II virus to human subjects three years after original feeding

Group No.	Status	Number of Subjects	Number Excreting Virus	Antibody Response
14	Fed before	19	10	16/19*
13	Control	3	3	3/3†

* Represents booster response.

† Represents primary response.

‡ Amount of virus fed approximates 100 human infectious doses.

Preliminary studies related to this problem were conducted four years ago (Koprowski *et al.*, 1952). Twelve subjects who developed Type II antibodies as the result of virus feeding were re-fed the same strain of virus after an interval of two to three months. The carrier state was again established in two of the 12. No significant rise in homotypic antibody was observed.

In the present trial, 19 subjects who had developed Type II antibodies following the oral administration of the TN strain three years before were re-fed the same preparation of virus, which had been kept frozen during the interval. The preparation represented an infected cotton rat brain and cord suspension. This, in contrast to the mouse adapted substrain of TN virus currently used, can be recovered from excreta, and the carrier state determined in a more accurate way. In addition to these 19 subjects (Table 5), three more individuals who had never been exposed to virus before and who had no antibodies against Type II received the same virus preparation. Since the same strain was used for titration purposes in man, it was possible to calculate that the inoculum consisted of approximately 100 human infectious doses. All three previously non-exposed subjects excreted virus in their stools and developed Type II antibodies. Ten of the 19 re-exposed individuals excreted virus again on one or more occasion. Stools of the remaining nine were found to be free from virus during a "collection" period of 30 days. The level of antibodies of re-exposed subjects who excreted virus ranged at the time of feeding from 1:4 to 1:64, as expressed by the protective titer of serum. The booster effect of the second virus feeding is illustrated in Fig. 3. Eight subjects were chosen for purposes of illustration, since their antibody titers had dropped about one log as compared to the values observed immediately following the first administration of virus. In spite of the fact that the antibody levels of the eight subjects were similar at the time of re-feeding, the incidence of intestinal carriage was not uniform after the second administration of virus: four of the individuals excreted virus and four did not. One may conclude that the appearance of virus in stools of re-exposed subjects is in no way related to existing levels of homotypic antibodies. Re-exposure to virus exercised, with one exception, a booster effect upon the antibodies, which returned to the levels observed after the first feeding of virus. Since the amount of virus administered to these individuals was not very large—representing only approximately 100 human infectious doses—it may be deduced that reinfection of the human intestinal tract with Type II virus may occur in subjects whose antibody titer is as high as 1:64.

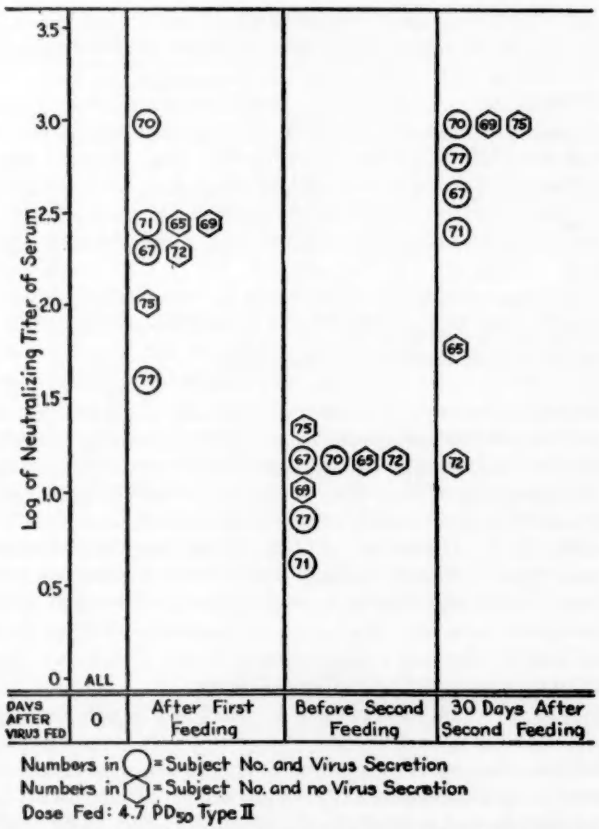

FIG. 3. Effect of refeeding of homologous strain upon level of antibodies against Type II.

"Permanence, persistence, is the first condition of all fruitful men."

Carlyle

Antibodies—development and persistence. In summarizing the conditions of acceptability of attenuated strains, according to the reaction of the human host (Koprowski, 1955a), I stressed the importance of the antibody response. There would be no sense in promulgating the idea of the use of living attenuated viruses as immunizing agents if we were not aiming at an antibody response in almost all subjects receiving virus. In our experience with the virus strains representing the two types, this phenomenon was actually observed, although—applying to ourselves the opinion expressed by A. J. P. Taylor about Toynbee—this may have occurred not because "of scholarship, but (because) of lucky dip, with emphasis on the luck." When the two types were administered simultaneously, the inter-

TABLE 6

Persistence of Type I antibodies in subjects fed SM strain

Subject	Titer of Antibody*	
	21 days	Months†
G	2.1	3.6 (14)
W	2.1	2.4 (14)
B	2.1	1.8 (17)
SC	1.1	1.2 (19)
JC	1.5	3.0 (20)
T	1.3	1.2 (17)
M	1.5	1.2 (19)

* Expressed as logs to base of 10.
† Figure in parentheses months after virus feeding.

ference phenomenon beset us on occasion, but in case of separate administration of the two strains, with the exception of a few children who regurgitated the virus immediately following its administration, all subjects developed homotypic antibodies. It is interesting to note that we failed to observe the development of heterotypic neutralizing antibodies in any of the trial subjects.

The data on the persistence of antibodies in subjects who received a single feeding of the Type I SM strain summarized in Table 6 encourage one to think that long-lasting immunity may be expected following live virus immunization. Similar observations of a four-year period of persistence of Type II antibodies (Koprowski, 1955b) following a single feeding of the TN strain (Table 7) add their weight to data compiled for the Type I strain.

If the presence of serum antibodies is an effective obstacle against paralytic poliomyelitis, then immunization with live virus in an attenuated form offers many advantages over any other method. In my opinion, the criteria of attenuation based on the apathogenicity of given strains for intracerebrally inoculated monkeys are now the best available in the laboratory. How "safe" these criteria are in relation to human pathogenicity can be—alas—known only in man him-

TABLE 7

Persistence of homologous antibodies after oral administration of Type II virus

Individual No.	Titer* of Type II Antibody†: Months after Administration of Virus	
	2–9	47–50
2	2.0	2.0
3	2.1	2.9
9	2.9	1.7
16	2.9	2.6
17	2.9	>3.4

* Expressed as log to the base of ten.
† Determined by pH color test in tissue culture.

self, the most susceptible species. If the number of subjects fed the same strain, with strictly defined characteristics, rises steadily without an accident then our confidence in the laboratory criteria of attenuation will be fortified.

The unfortunate persistence of alimentary infection with Type I virus may have to be averted. In contrast to the TN strain, the currently used Type I SM virus is highly cytopathogenic. Would it be possible that the latter characteristic determines the excessive rate of multiplication of the virus in the human intestinal tract? The development of a-cytopathogenic Types I and III would provide a welcome opportunity to test, at last, the hypothesis of "no cytopathogenicity, no excretion."

The source of material used for virus cultivation cannot be disregarded altogether. It should be represented by tissue which is least apt to harbor human pathogens—although the dilution factor which can be applied to a poliomyelitis virus suspension may be beneficial for the elimination of other "passenger" viruses.

The progress in research on live virus immunization is slow but steady. Its tempo depends unfortunately less and less on the actual achievements in the laboratory and in clinical trials, and more on the turbulent state of emotionalism which reigns at present in this field. Because of this, it is impossible at present to foresee the final outcome of the problem of immunization of man against poliomyelitis. It is also understandable that in such an age, the scientist bears a greater responsibility than ever. It is hoped that his voice will be listened to and that his judgment will prevail.

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PRESENT KNOWLEDGE OF THE CLINICAL SIGNIFICANCE OF THE ADENOIDAL-PHARYNGEAL-CONJUNCTIVAL GROUP OF VIRUSES

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The adenoidal-pharyngeal-conjunctival (APC) group of viruses (also called RI and ARD viruses) is a family of prevalent viral parasites of man causing catarrhal inflammation of the mucus membranes of the respiratory and ocular systems, with enlargement of submucus lymphoid tissues and regional lymph nodes. The viruses produce no recognizable illness in laboratory animals, but are studied by their cytopathogenic effects in tissue cultures of human and simian epithelium. In the brief period since their original recognition (Rowe *et al.*, 1953; Hilleman and Werner, 1954; Neva and Enders, 1954) certain representatives of the group have been established as the cause of a significant proportion of acute respiratory disease (ARD) of military recruits; a newly recognized clinical entity, pharyngoconjunctival fever; and sporadic cases of cararrhal and follicular conjunctivitis. Evidence is mounting which suggests that APC viruses also cause acute non-streptococcal pharyngitis, epidemic keratoconjunctivitis, and mesenteric lymphadenitis.

The purpose of this report is to review briefly the present knowledge of the pathogenicity and epidemiology of the APC viruses.

DEFINITION AND COMPOSITION OF THE APC GROUP

From the laboratory standpoint the identifying characteristic of the APC viruses is the soluble complement fixing antigen shared by all members, which is specific for the APC group. When used as antigen, each APC virus can be used to detect complement fixing antibody rises in paired serums of persons infected with any other APC type. In addition, viruses sharing this antigen have almost invariably demonstrated the common properties of ether resistance, production of characteristic cytopathogenic changes in tissue cultures of monkey kidney, human epithelium, and HeLa cells, and production of excess acidity in infected HeLa cell cultures (Rowe *et al.*, 1955).

Studies of the size of the virus particles indicated that virus present in culture fluids is spherical, with a diameter of 80 to 120 mmu (Hilleman *et al.*, 1955b), whereas intracellular virus, which is present in "crystalline-like patterns" in the nuclei of HeLa cells, measures 65 mmu in diameter (Harford, 1956, personal communication), or 30 by 50 mmu (Kjellén *et al.*, 1955). Filtration studies indicated that the soluble antigen is not larger than 26 to 40 mmu (Hilleman *et al.*, 1955b).

Serotypes within the APC group are defined by reciprocal neutralizing antibody titrations with rabbit hyperimmune serums (Rowe *et al.*, 1955; Rowe *et al.* in press; Kjellén, 1955). Fourteen serotypes have now been established, and

other APC viruses distinct from the established serotypes in one directional tests are still under study.

DISTRIBUTION AND PREVALENCE OF APC VIRUSES

All currently available information indicates that APC viruses not only are widespread in human populations but produce infections in all age groups. APC viruses have been reported from a number of geographical areas, including many parts of the United States, Canada (Ormsby *et al.*, 1955a), England (Zaiman *et al.*, 1955), Holland (Van der Veen, 1955, personal communication), Sweden (Kjellén, 1955), and Russia (Smorodintsev, 1956, personal communication). Complement fixing antibodies have been found in the serums of Egyptian children.

In the Washington, D.C. area, neutralizing antibodies to various APC viruses are highly prevalent in many age groups (Huebner *et al.*, 1954). Newborn infants generally had demonstrable complement fixing and neutralizing antibodies to a degree comparable to those of the mothers; by six weeks of age, the complement fixing antibodies disappeared, while neutralizing antibodies were still detectable. At one year of age, neutralizing antibodies were present only if complement fixing antibodies had again appeared, indicating that the neutralizing antibody detected at this time represented a response to infection (unpublished data). More than half of children tested between the ages of six and twelve months had neutralizing antibodies to at least one APC type, usually type 1 or 2. The incidence of antibodies to each type increased progressively with age, and of 50 persons 31 to 77 years of age, all had antibody to at least one type, and 72 per cent had antibody to four or more of the 6 types tested. Neutralizing antibodies to certain types are also prevalent in residents of Cleveland (Jordan *et al.*, 1954).

Infections with APC viruses are not restricted to human beings. Two viruses of the APC group, the S.V.$_1$ (Hull, 1955, personal communication) and Bertha (Sabin *et al.*, 1955, unpublished data) strains, were recovered only from simian sources. Also, complement fixing antibodies to the APC group have been found in the serums of normal chimpanzees and guinea pigs (unpublished data).

ASSOCIATION OF APC VIRUSES WITH ILLNESS

Respiratory infections in military recruits. Hilleman and Werner (1954) reported the first isolation of a virus of the APC group from persons with clinical illness, finding the RI-67 virus, the prototype of type 4, in cases of ARD and atypical pneumonia in military recruits (Hilleman *et al.*, 1955c). The role of type 4 in ARD was confirmed by Ginsberg *et al.* (1955a) who demonstrated specific neutralizing antibody responses to type 4 in paired serums of cases of ARD occurring during the 1940's, and of volunteers inoculated with filtrates of nasal washings from a case of ARD during the studies of the Commission on Acute Respiratory Diseases (1947). Hilleman and coworkers (1955a, d) performed extensive serologic surveys indicating the prevalence of APC infections in military recruit populations throughout the United States. Complement fixing

antibody rises to the APC group also occurred in military recruits in England and Holland (Zaiman *et al.*, 1955; Van der Veen, 1955, personal communication). Studies of the occurrence of APC viruses and of serological responses in naval recruits with both respiratory and non-respiratory illness served further to confirm Hilleman and Werner's original finding (Rowe *et al.*, to be published; Seal *et al.*, 1955, personal communication).

Hilleman *et al.* (1955e) pointed out the immunologic heterogeneity of the agents found in military recruits. APC types 3, 4, and 7 were recovered repeatedly from recruit respiratory illnesses including primary atypical pneumonia by Berge *et al.* (1955).

Thus, certain APC viruses are well established as the causative agents of a substantial proportion of acute respiratory disease (ARD), viral pneumonitis, and other related illnesses in military recruits. It should be pointed out, however, that the etiology of many, if not of the majority of undifferentiated febrile respiratory illnesses, even in this special population, remains undetermined.

Respiratory infections in the general population. Surveys for APC viruses and antibodies indicate that the most common and therefore probably the most important occurrence of APC viruses is in the general population, particularly in children, and that they are a significant cause of respiratory infections in non-military groups.

Evidence of APC group infection was demonstrated in 13 (25%) of 52 infants and young children with febrile respiratory diseases studied during the winter of 1953–1954 (Huebner *et al.*, 1954) and in 8 per cent of 168 respiratory illnesses in university students (Evans and Morse, 1955). The serotypes responsible for these infections were not determined.

APC type 3 is the cause of a newly recognized clinical entity, pharyngoconjunctival fever. The chief features of this disease, as observed in 114 virus positive cases (Parrott *et al.*, 1954; Bell *et al.*, 1955, unpublished data) are follicular conjunctivitis, pharyngitis, fever, cervical lymphadenopathy, and rhinitis. While frequently occurring together, the chief manifestations of the disease, conjunctivitis, pharyngitis, and fever, also occurred singly as well as in combinations. Asymptomatic infections were very rare. During 1954, this disease was observed in multiple localized outbreaks and sporadic cases involving several hundred persons in metropolitan Washington, D.C. Infections were observed to spread readily to a large proportion of susceptibles in households, a summer day camp, and on a hospital floor; swimming pools were suspected as contributing to the spread of infection. Attack rates were highest in children five to nine years of age, but many infections occurred in younger children and adults.

Retrospective serological surveys indicated that APC type 3 was also the cause of a 1951 epidemic of a similar disease entity, "Greeley Disease" (Cockburn, 1953; Cockburn, Rowe and Huebner, to be published). During this outbreak, about one fourth of the cases developed multiple transient corneal opacities.

During 1955, outbreaks clinically resembling pharyngoconjunctival fever occurred in Toronto (Ormsby and Aitchison, 1955) and England (Andrews,

1955, personal communication). Type 3 was demonstrated in specimens taken during similar outbreaks in Ohio (Anderson, 1955, personal communication) and New Hampshire.[1]

Ginsberg *et al.* (1955b) recovered nine strains of type 3 during a small outbreak of non-bacterial pharyngitis not associated with conjunctivitis, and Kjellén (1955) isolated two strains of type 3 from similar cases.

APC types 1, 2, and 5 have been recovered from cases of respiratory illness, but properly controlled epidemiological studies are required to determine their importance in the etiology of these illnesses. Prior to this communication, type 1 had been recovered from a case of exudative nasopharyngitis (Rowe *et al.*, 1955) and a fatal pneumonitis in an infant (Henle, 1955, personal communication), "Type II-like" virus from two cases of non-bacterial pharyngitis (Ginsberg *et al.*, 1955b), and type 5 from three cases of pharyngitis (Kjellén, 1955; Rowe *et al.*, 1955).

During the past two years, in collaboration with Drs. J. A. Bell, D. J. Davis, R. N. Philip, and K. K. Takemoto of this laboratory, we have conducted continuous virus isolation studies on cases of respiratory infections, chiefly in children of the Washington, D.C. area. To date, 6 strains of type 1, 17 of type 2, and 8 of type 5 have been recovered from throat or anal swabs in close temporal association with respiratory illness. The great majority of cases were febrile pharyngitis-tonsillitis or febrile coryza in children under four years of age. Of the children tested, the majority demonstrated complement fixing and neutralizing antibody responses to the recovered virus.

It must be emphasized that the isolations of serotypes 1, 2, and 5 represent observations on illnesses only, and that the simple associations of prevalent viruses with highly prevalent illnesses do not suffice to prove causation. However, the similarity of the illnesses associated with these serotypes to illnesses caused by types 3, 4, and 7 suggest that they are also pathogenic. The fact that, when swabbed on the palpebral conjunctiva, type 1 and 5 produced pharyngitis as well as conjunctivitis also supports this hypothesis (Bell *et al.*, unpublished data).

It is of interest that types 4 and 7, while highly prevalent in military populations, have not been recovered from illnesses in the general population; however, antibodies to these types are prevalent in adults with no military experience (Huebner *et al.*, 1954; Jordan *et al.*, 1954; unpublished data).

Ocular infections. Several APC serotypes are associated with infections of the conjunctiva and cornea. Many cases of catarrhal and follicular conjunctivitis due to type 3 have been observed, both as part of the clinical picture of pharyngoconjunctival fever and as simple conjunctivitis (Bell *et al.*, 1955; Ormsby *et al.*, 1955a; Ryan *et al.*, 1955). In addition, multiple transient superficial corneal opacities developed during the course of conjunctivitis apparently caused by type 3 in Greeley, Colorado (Cockburn, 1953), and Toronto (Ormsby *et al.*, 1955a, b). Similar opacities developed in the eyes of a volunteer after instillation of tissue culture fluids containing a type 3 strain recovered from a case of kera-

[1] Specimens received from Drs. G. Sobel and B. E. Aronson.

titis (Ormsby *et al.*, 1955a). On the other hand, opacities were never observed in volunteers receiving type 3 strains recovered from cases in the Washington, D.C. area, where keratitis did not occur (Ward *et al.*, 1955; Huebner *et al.*, 1955).

Jawetz *et al.* (1955) isolated a new APC serotype, type 8, from conjunctival secretions of a merchant seaman with epidemic keratoconjunctivitis (EKC). Furthermore, these workers demonstrated neutralizing antibody responses to this virus in each of seven cases of EKC, and found neutralizing antibodies in the convalescent serums of all of the 25 cases of EKC tested. In contrast, antibodies to type 8 were present in only two of 29 convalescent serums of patients with uveitis or herpetic keratitis. These observations suggest that this virus is important in the etiology of EKC.

Other APC viruses recovered from cases of conjunctivitis are type 6, found in two cases of catarrhal conjunctivitis in adults (Jawetz, 1955, unpublished data); type 10, the only isolation of which was from a child with granular conjunctivitis of the upper tarsal conjunctiva; and type 4, in a case of simple conjunctivitis in a laboratory worker (unpublished data).

It is of interest that swabbing the conjunctivae of volunteers with types 1, 3, 4, and 5 resulted in conjunctivitis indistinguishable from that observed in natural infections with type 3 (Ward *et al.*, 1955; Bell *et al.*, to be published).

Diseases of lymphoid tissues. A number of observations associate APC virus infections with involvement of lymphoid tissue. As described in a later section, these viruses are found in adenoids and tonsils of a high proportion of children undergoing tonsillectomy-adenoidectomy. A characteristic feature of acute infections with APC viruses, both in naturally acquired cases and in volunteers, is follicular enlargement of the submucus lymphoid tissue of the pharynx and conjunctiva, with enlargement of regional lymph nodes. The lymphoid nature of the conjunctival follicles was confirmed by biopsies of inoculated volunteers.

In addition, APC viruses have been recovered from several cases of more obscure diseases of lymphoid tissue. Rowe *et al.* (1955) recovered type 3 from the mesenteric lymph nodes and lungs of an infant dying of Letterer-Siwe disease, and subsequently the D.C. strain, was isolated from the stool of a fatal case of an undiagnosed reticuloendothelial system disease resembling Niemann-Pick disease (unpublished data). Kjellén (1955) isolated APC types 3 and 5 from lymph nodes or stools of children with mesenteric lymphadenitis.

Respiratory illness in volunteers. In studies by the Commission on Acute Respiratory Diseases (1947) mild, generally afebrile, illnesses were produced when filtered nasal washings from a case of ARD were inhaled. Retrospective serological studies by Ginsberg *et al.* (1955a) showed that the recipients of this nasal washing who developed illness also responded with specific neutralizing antibodies to the RI-67 strain (APC type 4).

Subsequent attempts to produce illness by nasal or pharyngeal inoculation of various viruses grown in tissue culture were negative or inconclusive (Huebner *et al.*, 1954; Hilleman *et al.*, 1955a). However, when virus was swabbed onto the conjunctivae of volunteers having no detectable neutralizing antibodies to

the inoculated type, easily recognized objective illnesses similar to pharyngo-conjunctival fever (Bell *et al.*, 1955) were produced with great regularity (Ward *et al.*, 1955; Bell *et al.*, to be published). Similar illnesses were produced by types 1, 3, 4, and 5, and were characterized by conjunctivitis and pharyngitis with submucus lymphoid follicular hyperplasia, coryza, and in some cases tonsillitis, regional lymphadenopathy, and fever. Persons having neutralizing antibody before challenge were almost uniformly resistant.

Persistence of APC viruses in adenoids and tonsils. During each of three years, APC viruses were isolated from a high proportion of adenoids or tonsils of children undergoing tonsillectomy-adenoidectomy (Rowe *et al.*, 1953, 1955). During 1954–1955, APC viruses were recovered from the adenoids and/or tonsils of 27 (90%) of 30 children tested. However, to recover virus, it was necessary to grow such tissues in culture; virus was recovered rarely from adenoid or tonsil tissue suspensions and not at all from throat swabs taken at the time of operation (Rowe *et al.*, 1955).

Although many isolates remain to be typed, strains of serotypes 1 through 6 have been recovered from such cultures; 61 (91%) of 67 strains typed belonged to types 1, 2, or 5. Type 4 was recovered twice, in both instances from adults. Type 3 was not recovered during studies from 1952 to the spring of 1954, when the virus apparently was not prevalent in this area, as indicated by neutralizing antibody surveys (Huebner *et al.*, 1954). However, in the winter of 1954 to 1955, following the appearance of this virus in epidemic form during the preceding summer (Bell *et al.*, 1955), three isolations were obtained. It is of note that the strains recovered from adenoid cultures in England were also types 1, 2, and 5 (Zaiman *et al.*, 1955; Andrewes and Pereira, 1954, personal communication).

Vaccine prophylaxis of APC infections. Preliminary studies of formalinized and heat-inactivated type 3 APC vaccines showed that such vaccines produced neutralizing antibodies in 35 (78%) of 45 vaccinees, with subsequent resistance to challenge inoculation with live virus. The protection afforded was comparable to that found in persons with naturally-acquired antibodies (Huebner *et al.*, 1955). Further studies are necessary to determine the value of APC vaccines in preventing natural illness and the duration of such protection.

Laboratory diagnosis. Laboratory diagnosis of APC group infections depends chiefly on two procedures, virus isolation and the complement fixation test. At present, HeLa cell cultures provide the most sensitive and convenient tissue for the recovery of most types. Roller tube cultures of human embryo epithelium would probably be required for isolation of the D.C. type, and monkey kidney cultures for the simian strain $S.V._1$.

The complement fixation test performed on paired acute and convalescent serums provides a simple, quick, and specific diagnostic procedure, but does not provide information as to the infecting serotype. In order to detect all complement fixing antibody rises, several antigens should be used. Neutralizing antibody rise is also indicative of infection, tending to be specific for the infecting serotype; because of the numerous serotypes in the APC group, this procedure is of limited value for diagnostic purposes.

SUMMARY

The newly recognized APC group of viruses consists of at least 14 serologically different viruses each sharing a common complement fixing antigen and having common biological properties. Many of these viruses are highly prevalent in the human population, and representatives also occur in certain animals. Types 1, 2, and 5 have been found most frequently in association with febrile respiratory infections in young children, and are unmasked in tissue cultures of adenoids and tonsils of most children undergoing tonsillectomy-adenoidectomy. Type 3 occurs in both civilian and military populations, where it causes a distinct disease entity, pharyngoconjunctival fever, sporadic cases of febrile pharyngitis and follicular conjunctivitis, as well as ARD. Types 4 and 7 cause a significant proportion of ARD and atypical pneumonia in military recruits. Types 6 and 10 have been isolated infrequently, and in association with conjunctivitis. There is evidence that type 8 may be the cause of epidemic keratoconjunctivitis.

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THE RICKETTSIAE: COMMENTS ON RECENT OBSERVATIONS ON BIOLOGY AND EPIDEMIOLOGY

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The purpose of this note is to stress the importance of certain findings in recent studies on rickettsiae and to comment on the implications.

BIOLOGY

Two groups of workers studying the virulence and activity of rickettsiae have obtained results of fundamental importance in the biology of these micro-organisms. Bovarnick and Allen (1954) found that typhus rickettsiae of the Madrid E strain lost their toxicity for mice, their hemolytic activity, their respiratory activity, and their infectivity for chick embryos as a consequence of freezing and thawing in isotonic salt solutions; they showed that these properties could be partially restored by incubation of the rickettsiae in the presence of diphosphopyridine nucleotide, DPN, and Coenzyme A for as short a time as two hours at 34°C. Bovarnick and Allen further showed that the inactivation and subsequent reactivation is quite sensitive to the presence of low concentrations of sucrose during the process. They followed this observation by making analyses on the concentration of DPN in rickettsial suspensions and were able to show that freezing and thawing in the absence of sucrose caused a change in the location of the DPN, allowing it to come out of the rickettsial cells into the supernatant fluid.

Such observations obviously mean that many previous experiments with typhus rickettsiae must be re-evaluated. The influence of various environmental factors on infectivity may be explained in part at least by events related to changes in concentration of DPN and Coenzyme A. Our concepts of dead as opposed to live rickettsiae now require sharper definition. The importance of various coenzymes in invasiveness of rickettsiae and in host-parasite relations has thus been opened for fruitful inquiry by the work of Bovarnick and Allen.

Price and Gilford (1955), working with Rocky Mountain spotted fever, RMSF, likewise found that DPN and Coenzyme A are of importance to the activity of these micro-organisms. It had previously been shown that RMSF rickettsiae exist under certain conditions in an avirulent phase in the tick *Dermacentor andersoni* (Price, 1953). Although this phase was avirulent for guinea pigs, the micro-organisms were able to multiply profusely in chick embryos. Price and Gilford showed that it is possible to convert the avirulent to the virulent phase by treatment of the micro-organisms in vitro with DPN and Coenzyme A. It was known that incubating infected ticks at body temperature for three days would result in conversion of avirulent to virulent phases. It was also known that the molting process of the arthropod had some control over this change in virulence. Price and Gilford investigated the conversion phenomenon

and found that incubation of RMSF rickettsiae with para-aminobenzoic acid resulted in loss of virulence and that this could be prevented by Coenzyme A and DPN. Further, they showed that the change in virulence was directly related to the ability of micro-organisms to adsorb to guinea pig cells in vitro.

These findings provide the basis for an explanation of the formerly mysterious activation in Rocky Mountain spotted fever. Price and Gilford's results certainly indicate that the virulence of this arthropod-borne micro-organism can be modified, not only by a metabolic product formed by the arthropod itself, possibly under hormonal control, but also by the presence or absence of critical amounts of para-aminobenzoic acid, DPN, and Coenzyme A.

EPIDEMIOLOGY

Recent observations on the recrudescence of epidemic typhus, occurring clinically as Brill-Zinsser disease, and of the persistence of various rickettsiae in man for long intervals after an attack of a rickettsial disease, are of potentially wide implication for infectious diseases in general. Murray and his colleagues (1951; 1953) have shown the validity of Zinsser's hypothesis that the recrudescence of epidemic typhus is the event which can initiate new outbreaks of typhus. Price (1955) showed that typhus rickettsiae persisted in lymph nodes of human subjects. Thus it is clear that the inter-epidemic reservoir of epidemic typhus is man.

Smadel and his associates (1952) had reported the persistence of scrub typhus rickettsiae in lymph nodes; Parker, Woodward and their colleagues (1954) demonstrated this phenomenon for Rocky Mountain spotted fever. Such observations suggest that this may be a common property of rickettsiae, namely, their ability to persist in the tissues for months or years after primary infection. Obviously this may be of considerable importance in explanation of the epidemiology of these diseases, particularly if it can be demonstrated that arthropods, feeding on man during mild, clinically unrecognized recrudescences, acquire rickettsiae from human blood and then can continue the chain of infection. This has been demonstrated in the case of human body lice and epidemic typhus, but it remains to be determined for scrub typhus and Rocky Mountain spotted fever.

The clinical course of rickettsial diseases is so profoundly altered by therapy with the tetracyclines or chloramphenicol that the identity of a recrudescence of any one of the various rickettsial diseases might be masked. This leads one to suspect that only a very alert physician, in cooperation with an active laboratory, will be able to make accurate diagnoses of recrudescent rickettsial infections. Despite such difficulties, it is postulated that the clinical counterpart of Brill-Zinsser disease will be found in the other rickettsial diseases in due course, albeit milder in severity and probably without rash.

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IMMUNIZATION AGAINST EPIDEMIC TYPHUS

A Brief General Review and a Description of the Status of Living, Avirulent *R. prowazeki* (Strain E) as an Immunizing Agent[1]

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In the same year that da Rocha-Lima (1916) described the agent of classical typhus in its louse vector and gave it the still current name of *Rickettsia prowazeki*, the first significant attempt at artificial immunization of man was made by Nicolle (1916) who used diluted blood from typhus patients. The numerous subsequent efforts to develop anti-typhus vaccines have followed one or the other of two fundamentally different approaches. Some have attempted to immunize with inactive or killed rickettsiae while others have tried to produce a safe but immunizing infection in man by means of living rickettsiae, either *R. prowazeki* itself or the closely related agent of murine typhus, *R. mooseri*. The present paper will review these developments with special attention to the more recent work, since there exist already a number of good reviews written in the period 1940 to 1948 (Murgatroyd, 1940; Biraud, 1943; Sadusk and Kuhlenbeck, 1946; Cox, 1948).

WORK WITH KILLED RICKETTSIAL VACCINES

Since an inactive agent by definition will not multiply in the host, a principal requisite of a killed-agent vaccine is that it contain sufficient original antigen to provide all the stimulus necessary for immunization. The story of the evolution of vaccines prepared with killed rickettsiae, therefore, is very largely the story of the search for suitable sources of abundant rickettsiae. Although there had been some earlier work, the first significant step was the discovery by da Rocha-Lima (1918) that the concentration of *R. prowazeki* in infected body lice was greater than that in infected human blood; he also claimed to have immunized guinea pigs by repeated injections of phenolized suspensions of infected lice. Weigl (1920) then described the intrarectal infection of body lice and Breinl (1924) reported the preparation of vaccines from the intestines of lice so infected. While the finally evolved method of producing louse-gut vaccine (Weigl, 1930) hardly lent itself to mass production, word-of-mouth reports indicate that in Russia during World War II vaccine was prepared on a large scale from triturated whole lice which had been infected in mass by feeding them on infected blood through a membrane of human skin taken from cadavers.

Several efforts to utilize tissues of infected animals (lungs excepted) did not yield results of significance and will not be described. Tissue culture, however,

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did offer real promise as a potential vaccine source. Although Nigg and Landsteiner (1932) had grown murine rickettsiae previously in Maitland-type cultures of guinea pig tissues, Kligler and Aschner (1933, 1934) were the first to cultivate *R. prowazeki* and to use such material after formalization for the reportedly successful immunization of animals. Subsequently, Zinsser and co-workers (1939) developed a method of cultivating rickettsiae in a solid agar-base tissue culture and reported (Zinsser *et al.*, 1940) the adaptation of this technique using chick embryo tissue for "mass production of vaccine against typhus fever of the European type." They reported that 3 workers could make in a week enough vaccine for 300 persons. Almost at once, however, two somewhat more practical sources of rickettsiae became available.

Castañeda (1939) reported that murine typhus rickettsiae, administered intranasally to such animals as mice, rats, rabbits and sheep, multiplied in great abundance in the lungs. Using such lung material as a vaccine, Castañeda (1941) claimed to have protected man against epidemic typhus. Soon thereafter, Durand and Sparrow (1940) and Durand and Giroud (1940, 1941) reported successful pulmonary infection with *R. prowazeki* in various rodent species and the production of experimentally effective lung vaccines. Also, Combiesco *et al.* (1941) reported a similar vaccine made from canine (puppy) lungs. However, in spite of evidence for their probable effectiveness in man, animal-lung vaccines have not enjoyed great popularity. Not only are they hazardous to produce but they possess a theoretically high reaction potential because of the extraneous animal lung protein which they necessarily contain.

The most widely used source of epidemic typhus rickettsiae for vaccine production has been the chick embryo. Although Zia (1934) cultivated both murine and epidemic rickettsiae on the chorio-allantoic membrane, it was not until Cox (1938) demonstrated rickettsial multiplication in the embryo yolk-sac that a really abundant chick-embryo source of typhus rickettsiae became available. Cox and Bell (1940) and Cox (1941) soon showed that formolized vaccines prepared from such material were effective in protecting guinea pigs. In 1942 Craigie (1945) described the use of ethyl ether in cleansing the vaccine of extraneous yolk material. With minor modifications, Craigie's method has become the standard one by which millions of doses of yolk-sac (Cox-type) vaccine against epidemic typhus have been produced during and since World War II.

Studies of the antibody response of man to vaccine (chiefly to Cox-type vaccine) constitute part of the evidence as to vaccine effectiveness and have served as a guide to vaccine dosage and to the number and spacing of inocula. Usually, determinations have been made of complement-fixing (CF) and neutralizing antibodies. After a relatively unsatisfactory response was observed following a 2-dose course of vaccine (Topping *et al.*, 1945) a course of 3 doses of 1 ml. each at weekly intervals was prescribed for the U.S. armed forces during the war. Later, as more potent vaccines were prepared, the recommended primary schedule was reduced to 2 doses with a 7–10 day interval. Fox and co-workers (1949), using murine typhus vaccine prepared by the Craigie method, reported that a single adequate dose (3.0 ml.) yielded results nearly equivalent to those follow-

ing the same amount of vaccine divided into 3 weekly doses. Finally, Murray *et al.* (1952) noted that a 2-dose course appeared to be more effective when the interval was 2 to 4 weeks rather than 7 to 10 days.

Duration of seroimmunity after primary immunization has not been fully studied. Available data indicate that CF antibody declines rapidly, often to undetectable levels (Topping *et al.*, 1945; Fox *et al.*, 1949; Fox *et al.*, 1954), but that neutralizing antibody is more persistent (Topping *et al.*, 1945). In 1944 Topping and co-workers (1945) described a dramatic response to a single booster inoculum given 9 months after the primary course; not only were the resulting antibody titers gratifyingly high but, in some contrast to the results of primary immunization, there was near uniformity of response. Further study of the "booster phenomenon" has yielded both confirmatory results and some additional information. Using murine typhus vaccine, Fox *et al.* (1949) observed better response at 50 weeks after the primary course than at 22 or 28 weeks. In the other direction, Gauld and Goodner (1951) and Murray *et al.* (1952) showed that the capacity to respond to a booster inoculum endured for at least 3 to 6 years. While several workers have reported the relatively rapid decline of CF antibody following the booster response (Topping, *et al.*, 1945, Zarafonetis, 1945; Murray *et al.*, 1952), the data of Topping *et al.* (1945) indicate that neutralizing antibody persists in relatively high titer for at least 9 months.

Evidence as to resistance of man to infection exists, but not in fully satisfactory form. One series of observations testifies to the protection afforded by vaccination to laboratory personnel handling virulent typhus rickettsiae. Weigl (1930) reported the absence of illness among workers vaccinated with louse-gut vaccine, who had been fed upon by large numbers of infected lice. Cox (1948) reported complete absence of disease among exposed personnel in a commercial laboratory who were given a primary immunizing course of 6 inoculations of Cox-type vaccine followed by reinoculation every 3 months. Other experiences, including that of the author and that described by Topping (1944), differed in that disease continued to occur in vaccinated personnel but with greatly reduced severity and no fatalities. This experience, however, was clouded because exposure to both murine and epidemic typhus often occurred simultaneously, and differentiation between the two infections is difficult in the vaccinated individual. In this connection, Murgatroyd (1940) cites instances described by Zinsser and from the Pasteur Institute in Tunis in which vaccination against one form of typhus (murine or epidemic) did not protect against infection with the heterologous agent.

Deliberate challenge of vaccinated persons has also been reported. Nicolle and Sparrow, cited by Murgatroyd (1940), reported that 2 children, vaccinated with Weigl vaccine, resisted infection with epidemic typhus rickettsiae. Other workers, however, have reported little decrease in morbidity after challenge but marked reduction in severity and near elimination of fatality. Such was the experience observed in Russia after 3 doses of Cox-type vaccine as reported by Ecke *et al.* (1945). Such also was the experience in the notorious large-scale prison camp experiment in Germany reported by Ding (1943) who used, however, an

obviously excessive challenge inoculum which caused 20 to 33 per cent fatality in 2 control groups and induced disease even in the vaccinated groups after an incubation period of only 2 to 3 days. Ding found that Weigl, rabbit-lung, and full strength Cox-type vaccines were about equally effective and better than a weaker Cox-type vaccine or one prepared from canine lung. The only mortality (0.5 per cent) occurred in those given the latter type of vaccine. More recently, Fox and co-workers (1955) produced clinical typhus in 2 normal controls and in 2 of 3 volunteers challenged at 9 months after a primary course of Cox-type vaccine. In a similar experiment, not yet reported, challenge at 21 months produced typhus in 1 of 3 volunteers. Severity of disease could not be evaluated because aureomycin was given in all cases after 48 hours of illness.

The effectiveness of vaccine in protecting against naturally occurring infection is most inadequately documented. Only 2 well-planned field trials have been attempted, one by Snyder in Spain in 1941 and one by Dyer and Topping in Bolivia in 1941–42; according to Ecke *et al.* (1945), both failed to yield definite results. However, some valuable data emerged from the World War II experience. According to Sadusk (1947), there were only 64 cases of epidemic typhus (all mild) in the entire U.S. Army in the period 1942–1945 during which time in contrast, 603 cases of murine typhus were observed. Long (1946) states that in early 1943 in North Africa there were only 2 mild cases of epidemic typhus among U.S. forces, whereas among equivalent numbers of unvaccinated Allied troops, there were 43 cases with 30 per cent fatality. Stuart-Harris (1945) reported 134 cases (33 per cent fatal) among British troops during January to September 1943 whereas in early 1944 after vaccination there were only 21 cases (14 per cent fatal). In Iran and Iraq Sachs (1946) reported 19 generally mild cases and 2 deaths in 51,000 vaccinated British troops who were particularly heavily exposed whereas, in the same period, there were 141 cases with 33 deaths among 155,000 unvaccinated but less exposed troops. Using the experience of employees of the Cairo Fever Hospital, Gilliam (1946) was able to show that 3 doses of vaccine (but not 1 or 2) was associated with a significant decrease in morbidity. Furthermore, clinical evaluation of the 61 cases in the vaccinated hospital personnel by Ecke *et al.* (1945) revealed a major reduction in severity of disease; the single fatality was in a patient becoming ill 3 days after the first dose of vaccine. These workers also noted that vaccine given during the incubation period appeared to have an ameliorating effect and therefore recommended its use in the face of an epidemic. Finally, another benefit of vaccination was pointed out by Snyder and co-workers (1949) who demonstrated the exceeding difficulty of infecting lice by feeding them on vaccinated patients.

EARLIER WORK WITH LIVING VACCINES

The numerous earlier attempts to immunize man against epidemic typhus have been reviewed elsewhere (Murgatroyd, 1940; Biraud, 1943; Sadusk and Kuhlenbeck, 1946; Perez Gallardo and Fox, 1948). The use of living *R. prowazeki* of unmodified, virulent strains was initiated by Nicolle (1916) who tried to employ minute doses. Subsequent workers employed various tactics such as coupling

the vaccine with immune serum (Weil and Breinl, 1923), "attenuating" it by cold (Nicolle *et al.*, 1927), the addition of oil and other substances (Balteanu and Constantinesco, 1937; Combiesco, 1937), or preliminary inoculation of "attenuated" living murine rickettsiae (Haagen and Crodel, 1944). Such efforts have been abandoned because usually either no immunity resulted or inoculation typhus was produced.

The living agent of murine typhus, *R. mooseri*, has been used extensively because of the more benign nature of murine typhus and the cross-immunity existing with typhus of the louse-borne type. Here again, various tactics have been employed to minimize the virulence of the agent including the use of unnatural routes of infection such as intranasal or conjunctival (Sparrow, 1935; Sparrow and Mareschal, 1939), "coating" the agent with egg yolk and oil (Nicolle and Laigret, 1935; Laigret and Durand, 1939), and treating the agent contained in guinea pig tissue or in flea feces with bile (Blanc, 1936; Blanc and Baltazard, 1937, 1940). Both the "coated" material and the biliated vaccine were used very widely in North Africa with claims by the French workers that the few cases of typhus observed (none serious) were in Europeans (Blanc, 1936; Blanc and Baltazard, 1940; Laigret *et al.*, 1937). However, when vaccines of the Blanc type (biliated flea feces) were used in other areas where much less prevaccination immunity may have existed, rather different results have been obtained. Vaccination in Chile in 1935 of some 800 persons is said to have resulted in grave illness in 23 per cent and 5 fatalities (Murgatroyd, 1940), while in Japan in 1943 it was observed (Sadusk and Kuhlenbeck, 1946) that 218 cases of typhus with one fatality occurred in one group of 290 vaccinated persons.

It seems certain that the various efforts at "attenuation" served chiefly to reduce the infectivity of the inocula rather than to attenuate the agent in the true sense of modifying the fundamental pathogenicity and virulence of the strains employed. Because much of the Arab population in North Africa may have been immune before vaccination, the favorable results reported by the French workers are outweighed by those reported from Chile and Japan. The conclusion, then, must be that, until a strain of typhus rickettsiae of demonstrated low pathogenicity for man becomes available, human immunization against typhus should be restricted to vaccines containing only inactivated rickettsiae. The following section will present evidence that such a strain of *R. prowazeki* now exists in the form of strain E.

WORK WITH LIVING, AVIRULENT R. PROWAZEKI (STRAIN E)

Since a detailed review of experience with strain E up to mid-1954 has been published only recently (Fox, 1955), the present account will be somewhat abbreviated except as it describes more recently available information.

Strain E originated in Spain in the hands of Clavero and Perez Gallardo (1943) during routine serial passage in chick embryo yolk-sac of one of several strains isolated during an epidemic in Madrid in 1941. Rather abruptly, during the 11th passage, this strain changed its character in the direction of lesser virulence for laboratory animals While the change has not been reproducible, the newly

acquired character of the strain has persisted through more than 300 further egg passages. Perez Gallardo and Fox (1948) confirmed and expanded the earlier observations in animals, and established the antigenic identity of strain E with unmodified strains of *R. prowazeki*. The most striking point of differentiation was the apparent inability of strain E to infect guinea pigs; not only was recovery of the strain from tissues impossible but a direct relation was found between the degree of seroimmune response and the number of rickettsiae inoculated.

Experimental infection of man was first undertaken in Spain. Although Clavero and Perez Gallardo (1944) reported the uneventful inoculation of more than 2,000 persons, all but 15 of these received lyophilized material, the infectivity of which was undetermined but probably too low to have induced human infection. No further infection of man was attempted until 1951 when a systematic effort was begun to evaluate strain E as a living agent for immunization of man against epidemic typhus.

In the first phase of this evaluation, carried out in the United States, 154 volunteers received living, strain E rickettsiae in varying amounts and by different routes and an additional 50 volunteers were given placebo material or commercially prepared Cox-type vaccine (Everritt *et al.*, 1954; Fox *et al.*, 1954; Fox, Jordan *et al.*, 1955). The strain E vaccine used in this and in all subsequent work consisted of appropriate dilutions of richly infected yolk-sac material lyophilized as a crude 10 per cent suspension in a special protective diluent (Bovarnick *et al.*, 1950). All material was carefully titrated for infectivity in embryo yolk sacs. The nature and frequency of reactions were determined by daily clinical observations. To study the development and persistence of immunity, sera collected prior to and at appropriate intervals after vaccination were tested for CF and, in many cases, neutralizing antibodies. In addition, at a number of postvaccination intervals, small groups of volunteers were tested for their ability to resist challenge with virulent *R. prowazeki*.

Reactions following strain E vaccination will be described in relation to the second phase of the evaluation but one may say at this point that none were sufficiently serious to contraindicate further work. Otherwise, several important observations were made: 1) the minimum dose of strain E necessary to immunize (and presumably to infect) man was found to be on the order of 4 log egg infective doses (E.I.D.); 2) it appeared that little or no invasion of the blood stream by strain E occurs; 3) as in the guinea pig, there was a definite correlation between number of rickettsiae inoculated and the maximum level of seroimmune response; 4) doses of 4.0 log E.I.D. or greater of strain E rickettsiae induced a detectable immune response with great uniformity (one failure in 146 vaccinations); and 5) better immune response followed intradermal than subcutaneous inoculation although in a limited trial the intramuscular route also showed promise.

Observations as to the duration of immunity have now been extended to 3 years. In Table 1 are presented the titers of CF and neutralizing antibody at various intervals after vaccination in sera from persons given larger (7.0 or 6.0 log E.I.D.) or smaller (5.0 or 4 log E.I.D.) doses of strain E vaccine. The relation

TABLE 1

Seroimmune status of volunteers bled at intervals up to 3 years after inoculation with strain E rickettsiae as indicated by titers of complement-fixing and neutralizing antibodies

Original Inoculum of Strain E (log E.I.D.)	Interval Post Inoculation (Months)	Number of Sera with Titers of 1 in X for											
		Complement-fixing antibody						Neutralizing antibody*					
		Number tested	<2	2-4	8-16	32	64+	Number tested†	0	1-2	3-5	6-11	12-23	24+
7.0 or 6.0	2	66	6	2	19	21	18	12					1	11
	6	64	1	5	31	14	13	12				1		11
	12	46	32	1	11	1	1	23			5	7	5	6
	24	29	13	12	1	3		26		2	7	5	5	7
	36	15	9	5	1			15		1		9	3	2
5.0 or 4.0	2	48	15	6	17	7	3	11			1	3	2	5
	6	44	10	8	22	2	2	11				4	5	2
	12	37	32	3	2			13			6	4	2	1
	24	15	13	1	1			15	1	4	6	1	2	
	36	10	9	1				9			3	3	2	1

* Sera tested in mice against from 1.5 to 3.0 toxic LD_{50} of infectious strain E yolk-sac suspension.

† Only a portion of the available sera taken at 2, 6 and 12 months were titrated for neutralizing antibody, and of the 24- and 36-month sera a few could not be tested because of insufficient quantity.

of initial serologic response of both types to vaccine dose is evident. CF antibody declines rapidly after 6 months and is only rarely present, and then in low titer, after 12 or more months. Neutralizing antibody also declines in the period between 6 and 12 months but persists thereafter at a relatively stable level, dropping below the level of detection in a single instance only.

Table 2 summarizes 5 experiments in which small groups of volunteers (including 2 normal controls in each case) were challenged intradermally with virulent *R. prowazeki*. These were conducted at intervals of from 2 to 36 months after primary immunization with strain E. Only one volunteer (in Experiment 1), of a total of 28 immunized with strain E, manifested even slight febrile response to challenge and this terminated spontaneously after 48 hours. In Experiments 4 and 5, there were also included volunteers who had received a primary course of Cox-type vaccine (2 doses of 1 ml. each with a 10-day interval) 9 or 21 months previously. Of the 6 men so vaccinated, only one completely resisted challenge; 2 men developed self-limited minor febrile reactions while the other 3 evolved as clinically typical cases of typhus as did all of the 10 non-immune controls who were included in the 5 experiments. All such cases were terminated promptly by aureomycin begun after 48 hours of illness. Table 2 also indicates the seroimmune status of the volunteers, in terms of titers of CF antibody just before challenge and at the period of maximum titer after challenge. Many volunteers who resisted the challenge clinically manifested a marked rise in titer of CF antibody. While a booster response to the small amount of antigen contained in the chal-

TABLE 2

Summary of results observed after challenge infections with virulent R. prowazeki *given to persons immunized with strain E or Cox-type vaccines and to normal controls*

Challenge Experiment Number	Immunization		Number of Volunteers		Number of Sera with Complement-Fixation Titers of 1 in X								
					Immediately pre-challenge					Maximum post-challenge			
	Type of vaccine	Interval (months)	Challenged	With febrile response*	<2	2–4	8–16	32–64	128+	2–4	8–16	32–64	128+
1	Strain E	2	6	(1)		1	1	3	1	1		2	3
	None	—	2	2	2								2
2	Strain E	7.5	6	0		3	3				1	4	1
	None	—	2	2	2							1	1
3	Strain E	12	6	0	3		3				5	1	
	None	—	2	2	2							1	1
4	Strain E	24	5	0	2	2		1			2	2	1
	Cox	9	3	2	3								3
	None	—	2	2	2							1	1
5	Strain E	36	5	0	1	4					4	1	
	Cox	21	3	1(3)	3							1	2
	None	—	2	2	2							1	1

* Figures in ( ) include persons developing slight fever of 1 or 2 days duration which disappeared without treatment; all other instances of febrile response evolved as clinically typical typhus and were terminated after 48 hours by treatment with aureomycin. Incubation periods in these latter cases were 11–12 days in Exp. 1, 6 days in Exp. 2, 9 days in Exp. 3 and 4, and 10–11 days in Exp. 5.

lenge inocula may explain some of the rises, it seems likely that in others challenge resulted in establishment of actual infection.

The foregoing data indicate that, as judged on serologic grounds, immunity induced by strain E may not prove to be of indefinite duration and hence may not be fully comparable to that following natural infection. However, both neutralizing antibody and effective resistance to virulent challenge appear to endure for at least 3 years.

For the second phase of the evaluation procedure the scene was shifted to Peru in and about the city of Arequipa. This and subsequent work in Peru has been done with cooperation of the Peruvian Ministry of Health and the Pan American Sanitary Bureau (PASB) and with the active participation of Dr. Juan A. Montoya, the regional typhus consultant for the PASB. Observations as to reactions and immediate serologic response to vaccination were materially expanded in order to define more specifically the most suitable infecting dose and route of inoculation and to gain some idea of the stability of the vaccine under field conditions (Fox, Montoya *et al.*, 1955). The first observations were made in some 2,200 civilians of all ages residing in the city of Arequipa or in several

small outlying communities. Later, sizable groups of soldiers were similarly observed. The basic plan involved collection of a prevaccination serum specimen in every case, inoculation by an appropriate route (intramuscular, subcutaneous or intradermal) with a placebo or with an appropriate dilution of strain E vaccine, close clinical observation for 21 days after inoculation in every case and collection of a single serum specimen at about 6 weeks postvaccination from as many persons as possible. All sera were titrated for CF antibody and all CF-negative sera, whether pre- or postvaccination, were screen-tested for neutralizing antibody to insure the recognition of all persons immune prior to vaccination and of all instances of low level response to vaccination. It is also pertinent that all of the vaccinations done so far in Peru were with material from a single large lot of vaccine which had an initial infective titer in eggs after lyophilization of $10^{-7.6}$, based on original wet weight of embryo yolk sac.

In Table 3, the results of testing the postvaccination sera are distributed in per cent form by CF titer and by route and dose of vaccine. Also shown is a figure for overall per cent of positive sera which includes any CF-negative sera found to have neutralizing antibody. Only those results are presented which relate to persons shown to have been non-immune before vaccination. The small differences related to route of inoculation seemed to favor the intramuscular route. Since, in addition, less annoying local reactions followed use of the intramuscular

TABLE 3

*Summary of seroimmune response to strain E vaccine in Peruvian population groups as measured by tests for cc plement-fixing and neutralizing antibody in sera collected 6 to 12 weeks after vaccination**

Vaccination Group	Route of Inoculation†	Per Cent Concentration of Yolk Sac	Number of Sera Tested	Per Cent of Sera with CF Titers of 1 in X					Overall Per Cent Positive‡
				<2	2-4	8-16	32	64+	
Civilians, Oct. 1953	IM	1.0	54		2	30	35	33	100
		0.1	50	12	4	30	22	32	94
	SC	1.0	53	4	4	26	38	28	96
		0.1	54	7	7	37	37	11	96
Army, April–June, 1954	IM	1.0	117	3	3	21	18	55	97
		0.25	95	7	4	21	23	44	96
		0.1	111	13	6	32	20	30	95
Civilians, Aug. 1954	IM	1.0	53	9	7	37	24	20	93
		0.25	30	7		40	27	27	93
Army, July 1955	IM	1.0	190	4	2	39	33	23	99

* Results are presented only for persons shown to have been non-immune at time of vaccination.

† IM—intramuscular; SC—subcutaneous.

‡ Figure for overall per cent positive includes sera without complement-fixing antibody but found to contain neutralizing antibody.

route, it was used exclusively after October 1953. The correlation between dose and level of serologic response is again evident. Perhaps the most important point, however, is that, with doses as small as 0.1 per cent yolk sac (estimated 4.0 to 4.5 log E.I.D.), immune response was observed in 95 per cent or more of those vaccinated. Also of interest is that use of the same lot of vaccine over a period of nearly 2 years from the time of preparation resulted in a very uniform pattern of response suggesting a high degree of vaccine stability.

Postvaccination reactions followed the same pattern in Peru as in the U.S. and only the much more abundant Peruvian data will be presented. These reactions fell into two principal categories, "immediate" and "delayed," which are most conveniently considered separately.

Immediate reactions included both local and systemic components and usually became evident between 12 and 36 hours after vaccination, reached their peak between 24 and 72 hours, and subsided by 96 hours. The occurrence of the principal manifestations are summarized in Table 4 in which it becomes evident that there is a marked correlation between dose of vaccine and frequency and severity of reactions. Indeed, because of such reactions, use of 10 per cent yolk-sac was curtailed rather promptly. Major interest lies in the fact that, with doses of 0.1 and 0.25 per cent yolk sac, definitely tolerable levels of reaction were observed. A number of points of interest, however, are not evident from the table. First, while route of inoculation had little influence on the systemic components of the reactions, it was related to the local manifestations. Intradermal inoculation was abandoned early in the study because, with doses of 1.0 per cent or more, there often developed at the site of inoculation marked redness and swelling with a central vesicle that went on to ulcerate and form an eschar. Also, the degree of local tenderness was found to be significantly less after intramuscular than after subcutaneous inoculation. Secondly, it was noted that neither age nor pre-existing immunity influenced the occurence of immediate reactions. This latter fact raised a question as to the nature of the factor responsible for these

TABLE 4

Summary of immediate reactions in groups vaccinated with strain E in Peru

Per Cent Concentration of Strain E Yolk Sac	Number of Persons Vaccinated	Per Cent of Vaccinated Persons (with)							
		Fever*	Head-ache	Nausea	Vomit-ing	Malaise	In bed†	Local reaction <+ ‡	Lymph-adenitis
10.0	101	64	71	17	7	61	7	54	42
1.0	951	30	49	9	3	29	2	30	21
0.25	252	5	23	1	1	14	1	29	14
0.1	955	5	17	2	1	8	0	7	7
Placebo	885	2	7	1	1	2	0	1	1

* Fever based on history of "fever and chilliness" or temperature readings in excess of 38°C.

† Confined to bed for at least 1 day.

‡ Local reactions were graded on the diameter of the area of redness and swelling, 1+ being equal to about 1 inch.

TABLE 5

Summary of delayed reactions in groups vaccinated with strain E in Peru

Per Cent Concentration of Strain E Yolk Sac	Number of Persons Vaccinated*	Per Cent of Vaccinated Persons (with)										
		Reactions							Fever	Headache	Nausea or vomiting	In bed
		Of all grades	Of grade†			Of days duration						
			1	2	3	1–3	4–6	7+				
10.0	83	21	7	10	4	8	2	10	7	19	4	2
1.0	754	29	21	8	1	15	10	4	10	29	3	1
0.25	121	19	12	6	1	12	6	1	3	18	1	1
0.1	724	26	19	6	1	12	11	3	5	25	3	1
Placebo	885	11	9	2	0	7	3	1	2	11	1	0

* Persons immune prior to vaccination have been excluded except in the placebo group since virtually no delayed reactions occurred in such persons.

† Reactions graded as follows: Grade 1, headache alone for any duration (unless very intense) or fever and headache or other symptoms for less than 4 days; Grade 2, intense headache alone, or other combinations of manifestations for 4 days or more; Grade 3, more seriously ill, i.e. confined to bed for several days.

reactions. Investigation indicated that, while the reaction-provoking property was inseparable from the rickettsiae, it could not be neutralized by relatively potent antiserum (Fox, Jordan *et al.*, 1955).

Delayed reactions usually began on or after the 9th postvaccination day and consisted chiefly of fever, headache, generalized aching and malaise. To assist in evaluating these reactions, a rough system of grading was devised which is described in a footnote to Table 5. The table itself summarizes the reactions observed with relation to dose of vaccine. Since these delayed reactions virtually did not occur in persons immune prior to vaccination, Table 5 presents data referable only to the 1,682 persons shown to have been non-immune. The most striking finding is that frequency and severity of the delayed reactions are not correlated with dose of vaccine. Allowing by simple subtraction for the reactions observed in the placebo control group, reactions attributable to the vaccine occurred in about 15 per cent of those vaccinated, being of grade 2 intensity in 4 to 8 per cent and of grade 3 in about 1 per cent. A final point, not evident from Table 5, is that delayed reactions were more frequent and more severe in older persons. Using figures not corrected for the occurrence of reactions in the placebo group, overall reactions and those of grade 3 intensity were observed in 26 and 1 per cent, respectively, of persons under 15 years of age and in 42 and 5 per cent, respectively, in those 30 years and older. While no treatment other than an antipyretic was offered in the present study, it seems probable that these reactions are manifestations of true infection with strain E rickettsiae and could be controlled by appropriate antibiotic treatment.

The final phase of evaluation of a vaccine should be the collection of evidence as to its effectiveness in preventing naturally occurring disease. This is now being attempted with reference to strain E vaccine in the Andean Indian population of Peru. Typhus is highly endemic in this population as is evident from

sero-surveys made in connection with this field trial (Montoya *et al.*, 1955). Overall, about 55 per cent are naturally immune with a distribution by age ranging from 8 or 9 per cent in the 0–4 year group to 94 per cent in persons 40 years of age and older. Although upwards of 15,000 persons have been vaccinated and another 7,500 have received placebo inocula, partly in October of 1953 and partly in August and December of 1954, an effective system of observation has been established only in recent months. While definite cases of typhus have been observed in the total study group, all in non-vaccinated persons, the data are not yet significant and final evaluation of the vaccine cannot be made as yet.

DISCUSSION AND SUMMARY

The evidence herein presented indicates that anti-typhus vaccines of the inactivated type are of well-established value. While most of the evidence relates to Cox-type vaccines of yolk-sac origin, the conclusions to be drawn probably are also applicable to potent vaccines derived from infected lice or animal lungs. It is clear that adequate primary immunization will greatly modify such disease as may occur and will virtually eliminate fatality. The degree of reduction in morbidity remains undetermined but is undoubtedly significant. Data as to duration of real immunity after primary immunization do not exist (our small challenge experiments failed to reveal solid immunity at 9 and 21 months post-vaccination). However, serologic evidence indicates that administration of single booster inoculum from 9 months to as long as 6 years later results in effective restimulation of immunity. While repeated booster inocula will undoubtedly serve to maintain a good level of immunity, an appropriate schedule of inoculations has not yet been worked out.

Use of a living rickettsial vaccine offers obvious possible advantages in that a single inoculation should result in solid and long-lasting immunity. Nearly all previous efforts to use living rickettsiae involved, basically, the use of minute doses of agents of unmodified virulence, minute because of dilution or partial inactivation. The concensus is that such vaccines, when given to non-immune persons, either failed to infect and to immunize, or produced unmodified, inoculation typhus.

The evidence available with respect to strain E indicates that it is indeed a strain of *R. prowazeki* of greatly reduced virulence for man. Its primary safety is attested by its use in upwards of 17,000 persons with no single known instance of truly serious illness resulting. Further, it is unlikely that it could be picked up and spread by lice because of its apparent inability to invade the blood stream. Admittedly, reactions do follow the use of strain E. However, there is a dose range (4.0 to 5.5 log E.I.D.) which induces both a tolerable level of reactions and a satisfactorily uniform immediate seroimmune response. Available evidence, with respect to both seroimmunity and ability to resist virulent challenge, indicates that, while immunity induced by strain E infection may not be fully comparable to that following natural infection, it is solidly effective for at least 3 years and probably considerably longer.

There are, however, some problems remaining. That actual infection of man

does occur is suggested on clinical grounds by the development of regional adenitis and of reactions of the delayed type. It also is suggested by the fact that the minimum immunizing dose of formol-inactivated rickettsiae is 2.5 logs greater than that of living strain E (Everritt *et al.*, 1954). However, knowledge as to the fact and possible sites of persistence of the agent in man should be sought. Also, while strain E-induced immunity seems superior to that following primary immunization with Cox-type vaccine, a comparison also should be made with that in persons given in addition a booster inoculum of inactive vaccine. Again, while the considerable field use of the vaccine in Peru testifies to its practical applicability under field conditions, criteria as to minimum infectivity, recommended dosage and expiration dates of production lots of vaccine remain to be defined. Finally, although the results of the challenge experiments offer much promise as to effectiveness, there remains the crucial problem of obtaining definitive data as to the effectiveness of strain E vaccine in preventing the natural occurrence of disease.

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SURVEY OF MOSQUITOES CAPTURED IN HONDURAS FOR YELLOW FEVER VIRUS

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An epizootic among the monkeys of the north coast of Honduras was reported beginning in December of 1953 and continuing into the late summer of 1954. The cause was assumed to be yellow fever and in two instances pathological confirmation of this diagnosis was obtained. Similar epizootics have occurred in other Central American countries in recent years (Elton, 1952; Yellow Fever Conference, 1955). Full details concerning this epizootic may be found in the report of Trapido and Galindo, 1955, and need not be repeated here.

Plans were made to capture and test mosquitoes from the affected area for yellow fever virus with a view to the identification of a possible natural vector. All mosquito collections were carried out by Drs. H. Trapido and P. Galindo, who have described methods of capture and location of the collecting sites in the report previously cited. Collections were made in August and early September of 1954. The mosquitoes were identified in the live state, pooled by species or species groups and shipped frozen in dry ice to the Gorgas Memorial Laboratory in Panama.

A total of 10,309 mosquitoes were received (See Table 1), the overwhelming majority (10,089) from the La Masica collecting station where a dead monkey with pathological changes characteristic of yellow fever was found on August 6. The remaining 220 specimens were captured at La Masica Arriba (208) and Lancetilla (12). A total of 15 different species or species groups were included, the two largest being the *Psorophora ferox* group with 5,657 specimens and *Trichoprosopon magnus* with 1,393 specimens. The only known natural vector of yellow fever received was *Haemagogus spegazzinii falco*, of which 14 specimens captured at La Masica Arriba were submitted for study.

The discrepancies between the numbers of mosquitoes given here and those reported in the paper describing the field work (Trapido and Galindo, 1955) are due to two factors: a small group of mosquitoes collected on the day before the routine collections were begun on August 8th was not included in the summary contained in the Trapido and Galindo paper; and small groups of mosquitoes of species not considered of possible significance in transmission, though received at the field laboratory alive, were not frozen for inoculation.

METHODS

The mosquitoes were triturated in groups of 50 or less with 10 per cent inactivated rhesus serum-saline which had been tested and found free of antibodies against yellow fever. Two cubic centimeters of the diluent were used for 50 mosquitoes and proportionately smaller amounts for smaller groups. The resultant suspension was then centrifuged at 5,000 r.p.m. during 15 minutes at

TABLE 1

Classification of mosquitoes collected in Honduras for virus studies

Place of Origin	Species	Total Number Received
Lancetilla	*Sabethes chloropterus*	12
La Masica Arriba	*Haemagogus spegazzinii falco*	14
	Psorophora cingulata	13
	Trichoprosopon magnus	100
	Trichoprosopon spp.	18
	Wyeomyia spp.	48
	Sabethes chloropterus	15
La Masica	*Haemagogus equinus*	498
	Aedes serratus group	162
	Aedes angustivittatus	21
	Aedes (*Ochlerotatus*) spp.	918
	Mansonia spp.	710
	Psorophora ferox group	5,657
	Trichoprosopon magnus	1,393
	Trichoprosopon spp	16
	Wyeomyia spp.	55
	Sabethes cyaneus-tarsopus	77
	Sabethes chloropterus	235
	Sabethini	343
	Chagasia bathanus	4
	Total	10,309

Aedes (*Ochlerotatus*) spp. includes: *A. serratus*, *A. tormentor*, *A. hastatus*, *A. oligopistus* and *A. angustivittatus*.

Psorophora ferox group includes: *P. ferox*, *P. lutzii* and *P. varipes*.

Mansonia spp. includes: *M. indubitans*, *M. titillans*, *M. venezuelensis*, and *M. nigricans*.

Sabethini includes: *Wyeomyia* (*Wyeomyia*) spp., *Wyeomyia* (*Dendromyia*) spp., *Trichoprosopon longipes*, *T. espini*, *T. fluviatilis*, *Limatus durhamii* and *Limatus asulleptus*.

a temperature of 5°C. To that part of the supernatant fluid destined for intracerebral mouse injection was added a concentration of 600 Units of penicillin and 600 micrograms of streptomycin per cubic centimeter, in order to control the bacterial contamination. Antibiotics were not added to the part destined for monkey inoculation.

Six young adult Swiss white mice were injected intracerebrally per specimen. The mice were kept under close daily observation for a period of 30 days. Any mice found dead, or sacrificed when showing signs of illness, were autopsied and their brains removed for direct smears, cultures and subinoculations in mice. Mice surviving throughout the observation period were challenged for immunity by the intracerebral injection of 100 LD_{50} of French neurotropic virus.

Rhesus monkeys, *Macaca mulatta*, were used for attempted virus isolation from four species or species groups: *Haemagogus equinus*, *Trichoprosopon magnus*, *Aedes* (*Ochlerotatus*) spp. and *Mansonia* spp. These species were selected since

epidemiological evidence seemed to incriminate them most strongly. Monkeys were inoculated subcutaneously with the pooled residual supernatant solutions of the centrifuged mosquito suspensions after the part destined for mouse inoculation had been separated. Monkeys were kept under observation for a period of one month and their temperatures were taken twice daily. At the end of 2, 4 and 6 week intervals after inoculation the monkeys were bled and their serums tested for yellow fever antibodies. Both the intracerebral and intraperitoneal mouse protection test techniques were employed.

RESULTS

No yellow fever virus was recovered from any of the 10,309 mosquitoes examined either in mice or in rhesus monkeys. Mice failed to show immunity when challenged at the end of their observation period with French neurotropic virus and the monkeys failed to develop neutralizing antibodies in their blood.

PSOROPHORA VIRUS

Although yellow fever virus was not recovered during this study, an unidentified neurotropic virus was isolated in mice from 50 specimens of a group of *Psorophora* captured at La Masica on September 2, 1954. The group included *Psorophora ferox*, *P. lutzii* and *P. varipes*. This virus has been maintained for 15 continuous passages to date in mice. No other susceptible animal has as yet been found. Cross-immunity experiments in mice and protection tests using known positive antiserums have demonstrated that this is not a strain of yellow fever virus. The following evidence indicates that it is not a spontaneous virus of our stock mice. Spontaneous infection with a similar virus has never been observed in our stock mice in the course of extensive experimentation. All mice inoculated with passage virus have developed the disease indicating the absence of immunity in the stock. Reinoculation of mice with the residue from the original mosquito suspension after 20 days of storage at −15°C. resulted in a second isolation of the same virus. Further studies of this agent are being conducted and will be published later.

CONCLUSIONS

Yellow fever virus was not recovered from 10,309 mosquitoes captured in Honduras in the late summer and early fall of 1954.

A neurotropic virus immunologically distinct from yellow fever was isolated in mice from one group of 50 *Psorophora*.

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ANTIBIOTIC DIETARY SUPPLEMENTS IN THE THERAPY OF CHILDHOOD PROTEIN MALNUTRITION

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During the course of the studies herein reported, a complete review of the literature on kwashiorkor was published (Trowell *et al.*, 1954). This monograph covers the older literature as well as the recent work, and especially the theories and practices carried out in the clinics and laboratories located in Kampala, Uganda. Trowell *et al.* have clearly demonstrated the similarity of various types of protein malnutrition in different parts of the world. They have stressed the need to differentiate this syndrome from that of caloric undernutrition and have alluded to the qualitative as well as the quantitative nature of the protein deficiency of the diet which leads to the development of kwashiorkor. The divorce between pellagra and kwashiorkor is emphasized but the authors have suggested instead that vitamin B_{12} plays a role in the etiology and therapy of kwashiorkor. The importance of changes in the liver, although they have been carefully recorded, has been minimized and instead, stress has been laid on the changes in the exocrine enzyme secreting glands, especially those of the pancreas. The suggestion is made that mild cases of this type of deficiency may be widely prevalent but difficult to diagnose. They stress the importance of animal protein, especially skim milk, and of mixed vegetable proteins supplemented with vitamin B_{12} in treatment. For the assessment of other factors they suggest that these be tried out in treatment at fixed levels of protein intake!

A review of kwashiorkor in India has also been recently compiled (Gopalan and Ramalingaswami, 1954). These authors bring out the fact that cases studied at Madras and Coonoor in India differ from those described in Africa by virtue of a high incidence of ascaris infection, a lower incidence of skin changes and a greater amount of vitamin A deficiency. Furthermore the Indian cases showed a tendency to relapse often with fatal outcome.

The review article first cited refers to some rather disturbing findings in cases of kwashiorkor. One of these discrepancies is that despite the beneficial effect of skim milk therapy there is a persistence of a moderate degree of anemia (Adams, 1954) and a failure of this anemia to respond to conventional therapy (Pieraerts, 1949). Another disturbing feature is the presence of an increased amount of gamma globulin in the serum of some cases of kwashiorkor whether the plasma proteins are separated chemically (Dean and Schwartz, 1953) or electrophoretically (Anderson and Altmann, 1951). Furthermore during treatment with skim milk there was a further rise in the gamma globulin level to even a higher figure (Dean and Schwartz, 1953).

In the treatment of kwashiorkor by the methods that have been outlined, the

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return of serum albumin to normal is sometimes incomplete and sometimes delayed (Adams, 1954; and Holmes, 1954). In severe cases the patient may not survive despite prompt and energetic treatment (Walt *et al.*, 1950; Dean and Schwartz, 1953; and Adams, 1954).

It is not surprising that the reviewers (Trowell *et al.*, 1954) should have alluded to the fact that it may be necessary to look elsewhere than to the total protein, methionine, vitamin B_{12} or choline in the search for factors causing or curing kwashiorkor. In fact they have quoted P. Gyorgy (1952, personal communication) who suspected that in skim milk there was some substance with protein sparing action.

The cases reported from India also showed a persistent low grade anemia, a high globulin that rose even more during treatment, and an albumin concentration after intensive therapy that remained lower than that found in normal children who had ordinary home diet. In some cases transfusions were thought necessary to prevent a high mortality. (Gopalan and Ramalingaswami, 1954).

Our approach to this problem was quite different. First an investigation was made into the normal plasma protein pattern of Indian students (Satoskar and Lewis, 1954 a). This revealed an abnormal elevation of gamma globulin and of total globulin which was unrelated to any evidence or history of illness except for a correlation with lower than normal hemoglobin levels and with the history of previous attacks of anemia. It also revealed a correlation with diet showing a tendency for higher globulin to be present in those who ate large amounts of vegetable protein and for a lower globulin in those who ate more of meat, milk or curds. Probably the diet can in part account for the discrepancy between the serum protein pattern of the Indian students and individuals resident in temperate zones.

Our next step was to see if the elevated serum globulin would return to normal through the use of animal protein factor (Gaitonde, 1954), vitamin B_{12} (Satoskar and Lewis, 1954 b) or antibiotics (Satoskar and Lewis, 1955). It was concluded as a result of these three studies that small doses of the broad spectrum antibiotics, chlortetracycline[2], oxytetracycline[3] and tetracycline[4] could have a potent action, even in so called normals, in causing a fall in the serum globulin and a rise in serum albumin. Furthermore in these experiments the amount of antibiotic used was only 50 mg. daily which is approximately one-fortieth of the amount usually prescribed in the treatment of infectious diseases.

Although anxious to investigate the mechanism of this phenomenon we felt that it was more urgent to see to what extent antibiotic therapy would modify the course of protein malnutrition and especially if it would aid in the treatment of clinical cases. The results of such studies form the basis of this report.

METHODS

To evaluate the effect of antibiotic nutritional supplements upon the therapy of protein malnutrition, it was necessary to run a control series along side of the

[2] The trade name of Lederle & Co. for chlortetracycline is Aureomycin.
[3] The trade name of Pfizer & Co. for oxytetracycline is Terramycin.
[4] The trade name of Lederle & Co. for tetracycline is Achromycin.

treatment group. No effort was made to restrict the patients to classical cases of kwashiorkor since we were interested to know in general what type of case might be benefited by antibiotics. The therapy in both series was similar except that one series received oral antibiotic and the other did not. Two girls who did not respond well to the routine treatment after eight weeks of hospitalization were then given antibiotics in addition and appear in this series both as controls and treatment cases (C2 and R2 and C9 and R9).

The diet used in the hospital contained about 1,400 calories and about 54 grams of protein including 12 ounces of cow's milk daily and two ounces of casein hydrolysate. Multivitamin drops or multivitamin tablets were also administered.

The antibiotic dietary supplement was given orally once daily as 25 milligrams of chlortetracycline or oxytetracycline. A liver biopsy was attempted on all patients and the course of the disease was followed by means of determination of the hemoglobin, red blood cell count, hematocrit and total and fractional plasma protein. These were usually estimated after four and eight weeks of treatment.

The technique used for estimating total protein and for electrophoresis has been described elsewhere. (Satoskar and Lewis, 1954 a; 1954 b).

RESULTS

From inspection of Table 1 it may be seen that the two series of cases, those receiving conventional therapy and those receiving conventional therapy plus antibiotics, were very similar. From inspection of Table 2, it will be noticed that somewhat more intensive therapy was given to those patients who were not receiving the antibiotics. Thus in the control group seven patients were given an average of 12 injections of liver extract plus vitamin B, while in the series of cases receiving antibiotics only 4 patients received an average of 4 injections of liver extract plus vitamin B. Four of the patients in the control series were given iron either orally or intravenously while none of the patients in the antibiotic therapy group were given iron.

Several patients in each series who showed deficiency either of vitamin A or nicotinic acid were treated with these. It will be seen that some patients in both series were followed for a while at home, where the diet could not be controlled. Shortage of hospital beds was in part responsible for this modification in our procedure.

The effects of therapy upon the hematocrit, red cell count and hemoglobin and upon the serum protein fractions are given in Table 3 for the patients on conventional therapy and in Table 4 for the patients receiving antibiotics. The results are most easily compared by plotting the values for these blood constituents against the number of days of treatment. We have selected 3 of the possible variables, namely the hematocrit, the plasma albumin and the plasma gamma globulin.

Chart 1 gives the initial and final values for the red blood cell volume of those cases that were followed for a period of 34 days or longer. Inspection of the graph shows that most of the cases treated with antibiotics have a more rapid rate of increase in red blood cell volume. In fact the control cases achieved

TABLE 1

Clinical character of the cases

Case Number	Sex	Age, Years	Home Diet	Edema	Vit. A Lack	Vit. B Lack	Skin Change	Anemia, Degree	Fatty Liver	Intestinal Parasites
C1	Male	1¼	Mixed	—	—	—	Yes	Moderate	Slight	—
C2	Fem.	3	Mixed	Yes	Yes	—	—	Moderate	Severe	*Ascaris* 1.
C3	Male	1¼	Veg.	Yes	Yes	—	—	Marked	None	—
C4	Male	2½	—	Yes	Yes	—	—	Slight	—	—
C5	Fem.	3	—	Yes	—	—	—	Moderate	—	*Ascaris* 1.
C6	Fem.	2	Veg.	Yes	—	—	—	Moderate	—	—
C7	Fem.	3¼	Veg.	—	Yes	—	Yes	Moderate	—	—
C8	Male	1¼	Veg.	Yes	—	—	—	Moderate	—	—
C9	Fem.	7	Veg.	Yes	—	Yes	Yes	Moderate	—	—
C10	Male	4	—	Yes	Yes	—	Yes	Moderate	Marked	*E. histolytica*
R1	Male	½	Veg.	—	—	—	—	Moderate	Marked	—
R2	Fem.	3	Mixed	—	—	—	—	Moderate	—	—
R3	Fem.	5	Veg.	Yes	Yes	—	—	Moderate	None	—
R4	Male	2½	Veg.	—	—	—	—	Moderate	—	—
R5	Male	3	Veg.	Yes	Yes	—	Yes	Moderate	Severe	—
R6	Fem.	3	Veg.	Yes	Yes	—	Yes	Moderate	Slight	—
R7	Male	2½	Veg.	—	—	Yes	—	Moderate	None	—
R8	Fem.	2½	Veg.	Yes	Yes	—	—	Moderate	Marked	—
R9	Fem.	7	Veg.	—	—	—	Yes	Moderate	Slight	—
R10	Male	3	Veg.	Yes	Yes	—	Yes	Severe	Slight	*Ascaris* 1.

C2 became R2 and C9 became R9, that is they were first control cases and later treatment cases.

hematocrit levels which vary between 22 and 35 per cent and average 30 per cent whereas the final values in the antibiotic treatment cases vary between 29 and 42 per cent and average 37 per cent.

Inspection of Chart 2 shows the difference in behavior of the plasma albumin level in the two series of cases. Those patients which did not receive antibiotics showed a rise in the plasma albumin when the initial level was very low but when the level was between 2.5 and 3.5 gm. per 100 ml. there was a tendency for the level to remain constant or fluctuate only moderately. On the other hand, all the cases receiving antibiotics showed a rise in the plasma albumin level although this rise was more extreme when the serum protein level was low to start with. After treatment, the plasma albumin level in the control cases varied between 2.1 and 3.4 gm. per 100 ml. and averaged 2.7 gm. whereas the cases treated with antibiotic showed a plasma albumin level of between 2.8 and 4.0 gm. per 100 ml. averaging 3.6 gm.

Inspection of Chart 3 shows what happened to the total globulin during the period of hospitalization. This chart is somewhat confusing because some of the subjects showed a rise in globulin and some a fall. However among cases treated with antibiotics there was a fall in globulin in most individuals who started with levels higher than normal (European or American standard). The final level of

gamma globulin after therapy in the antibiotic cases averaged 1.6 gm. compared with 1.8 gm. in the controls.

From the clinical viewpoint it was difficult to compare progress of cases in the two series although some of the individuals receiving antibiotics seemed to show a more favorable response. This was especially evident in the cases of kwashiorkor (compare R5 and C10). Those children who received antibiotic were the first to regain appetite, to smile, to get out of bed and remain at play.

A noticeable difference in the two series was the occasional recurrence of diarrhea in the control cases and the rarity of such phenomena in the patients re-

TABLE 2

Synopsis of the treatment given to cases in this study

Case Number	Hosp. Diet, Weeks	Home Diet, Weeks	Liver Vit. B Inject.	Nicotinic Acid	Vitamin A	Iron Therapy	Penicillin, Weeks	Sulfa-Drugs, Weeks	Other Therapy	Antibiotic Used
C1	7	0	16	—	—	Yes	—	—	Folic acid	—
C2	7	0	0	—	Yes	—	3	—	Hetrazan	—
C3	4*	0	9	—	Yes	—	2	2	Transfusion	—
C4	4	3	13	—	—	—	—	1	—	—
C5	4*	0	11	—	—	Yes	—	1	—	—
C6	4*	0	5	—	—	Yes	—	—	Prot. hydrol.	—
C7	4	3	0	—	—	—	—	1	—	—
C8	3	6	15	—	—	—	2	—	Oxycide	—
C9	8	0	14	Yes	Yes	—	3	1	Atabrine	—
C10	4†	2	0	—	Yes	—	2	1	Vioform	—
R1	8	0	0	—	—	—	1	—	—	Oxytetracycline
R2	7	0	0	—	—	—	—	—	—	Oxytetracycline
R3	7	0	0	—	Yes	—	1	—	—	Oxytetracycline
R4	5	0	6	—	—	—	1	—	—	Chlortetracycline
R5	8	0	2	—	Yes	—	—	—	—	Oxytetracycline
R6	4	5	2	—	Yes	—	—	—	—	Chlortetracycline
R7	2	7	0	—	—	—	—	1	—	Oxytetracycline
R8	4	3	0	—	Yes	—	—	—	—	Chlortetracycline
R9	0	7	0	—	—	—	—	—	—	Chlortetracycline§
R10	3‡	0	3	—	—	—	1	—	Transfusion	Chlortetracycline

* Died in the Hospital.
† Discharged due to lack of beds.
‡ Left against advice.
§ Received the medication only four weeks.

TABLE 3

Hematocrit, red cell count, hemoglobin and plasma protein levels of cases receiving conventional therapy

Case Number	Days of Therapy	Hematocrit	R.B.C. Count, Million	Hemoglobin	Albumin	Globulin Alpha 1	Globulin Alpha 2	Globulin beta	Globulin Gamma	Total Globulin
		%		gm. %	gm. %	gm. %	gm. %	gm. %	gm. %	gm. %
C1	0	32	2.5	8.0	3.26	.24	.46	.63	2.42	4.15
	21	33	3.8	9.0	3.25	.38	.71	1.10	1.94	4.13
	45	35	3.7	9.8	3.00	.29	.59	1.00	1.81	4.69
C2	0	29	2.8	7.5	1.39	.52	.52	.62	1.05	2.71
	27	34	2.8	10.0	2.40	.45	.60	.62	1.74	3.41
	56	32	3.1	9.8	2.80	.24	.51	.68	2.79	4.22
C3	0	27	2.1	8.0	0.68	.34	.42	.34	1.02	2.12
C4	0	30	3.6	11.6	3.30	.12	.24	.73	2.20	3.29
	24	33	3.7	12.4	3.38	.13	.40	.70	1.69	2.92
	41	33	3.5	12.2	3.37	.20	.46	.49	1.50	2.65
C5	0	29	2.6	9.0	1.03	.23	.34	.46	1.14	2.17
C6	0	30	3.2	10.0	1.15	.12	.24	.75	1.14	2.25
C7	0	32	3.5	7.6	2.24	.36	.47	.50	1.53	2.86
	31	30	3.5	9.0	2.76	.27	.50	.50	0.90	2.27
	51	35	3.6	10.5	3.24	.22	.53	.64	1.26	2.65
C8	0	29	2.7	7.4	1.37	.10	.31	.42	2.00	2.83
	21	30	3.0	7.5	3.30	.10	.20	.80	1.70	2.80
	63	30	2.9	7.6	2.40	.24	.80	.90	1.66	3.60
C9	0	30	2.4	8.2	2.53	.12	.29	1.20	2.36	3.97
	35	28	2.6	8.1	2.85	.14	.54	1.05	2.92	4.65
	55	28	2.7	8.0	2.73	.28	.61	.96	2.42	4.27
C10	0	32	3.8	12.0	2.04	.43	1.12	.60	2.31	4.46
	27	22	3.0	9.0	2.14	.32	.64	.82	2.08	3.86
	44	32	3.6	9.5	2.70	.30	.50	1.00	2.00	3.80

ceiving small doses of antibiotics. It was not necessary to give sulfa drugs to control diarrhea except once in this group, see Table 2. The weight records were not analysed because edema on admission distorted the picture. When the liver biopsy showed severe fatty infiltration this reverted to normal during the period of 8 weeks on antibiotic therapy, but on conventional therapy this was not always so, as in case C9.

Three individuals in the control series died after 30, 27 and 25 days in the hospital. The initial albumin level in those cases was 0.68, 1.03 and 1.15 gm. per 100 ml. However in the antibiotic series there were cases with initial albumin levels of 0.87, 1.03 and 1.39 gm. per 100 ml. In one of the control cases death was

TABLE 4

Hematocrit, red cell count, hemoglobin and plasma protein levels of cases receiving antibiotic in addition to conventional therapy

Case Number	Days of Therapy	Hematocrit	R.B.C. Count, Million	Hemoglobin	Albumin	Globulin Alpha 1	Globulin Alpha 2	Globulin Beta	Globulin Gamma	Total Globulin
		%		gm %	gm. %	gm. %	gm. %	gm. %	gm. %	gm. %
R1	0	35	3.2	10.0	3.75	.27	.80	.80	1.88	3.75
	25	35	3.2	9.0	3.57	.35	.66	.71	2.15	3.87
	42	39	3.4	11.0	4.00	.39	.47	.70	2.24	3.80
	56	39	3.9	12.0	4.00	.33	.59	.63	1.42	2.97
R2	0	32	3.1	9.8	2.80	.24	.51	.68	2.77	4.20
	48	40	3.8	11.0	3.90	.10	.29	.81	1.38	2.58
R3	0	27	2.0	7.7	0.87	.20	.34	.40	1.70	2.64
	31	29	2.2	8.0	2.60	.20	.69	1.18	2.60	4.67
	48	40	3.5	12.0	3.90	.38	.47	.71	1.82	3.38
R4	0	35	3.3	7.8	3.20	.34	.49	.98	1.99	3.80
	34	41	4.2	12.4	3.40	.34	.50	.77	1.69	3.80
R5	0	27	2.6	8.0	1.03	.32	.54	.46	1.65	2.97
	31	38	3.8	12.5	3.50	.20	.25	1.02	2.03	3.50
	52	42	4.2	13.0	3.70	.15	.30	1.08	1.78	3.31
R6	0	29	3.0	8.4	1.80	.42	.80	.87	1.61	3.70
	31	28	3.0	8.5	2.50	.37	.80	.98	1.35	3.50
	50	29	3.4	—	3.40	.32	.38	1.20	2.20	4.00
	65	29	3.7	8.0	3.90	.10	.50	.65	1.35	2.60
R7	0	30	3.1	10.0	2.20	.19	.20	.49	1.20	2.08
	31	33	3.8	11.4	3.80	.16	.39	.55	1.10	2.20
	61	37	4.0	13.5	4.00	.19	.45	.66	0.90	2.20
R8	0	28	2.6	8.4	1.31	.30	.41	.98	2.00	3.69
	28	29	3.2	9.0	3.24	.31	.60	1.10	1.82	3.83
	46	33	3.3	8.5	4.20	.20	.60	.60	1.68	3.08
R9	0	28	2.7	8.0	2.73	.28	.61	.96	2.42	4.27
	47*	31	3.6	9.0	3.40	.20	.50	.70	2.20	3.60
R10	0	12	1.9	3.6	2.14	.24	.47	.63	1.22	2.16
	21	39	4.5	11.0	2.77	.41	.41	.89	1.22	2.93

* On antibiotic for only 26 days.

sudden and unexplained, in one it was due to infection at the site of injections and in one case the patient had a heavy ascaris infestation.

TYPE OF CASE AND RESPONSE TO ANTIBIOTIC

The cases included in this study covered a wide variety of types. There was one case of typical pellagra, but this individual, C9-R9 also had hypoproteinemia

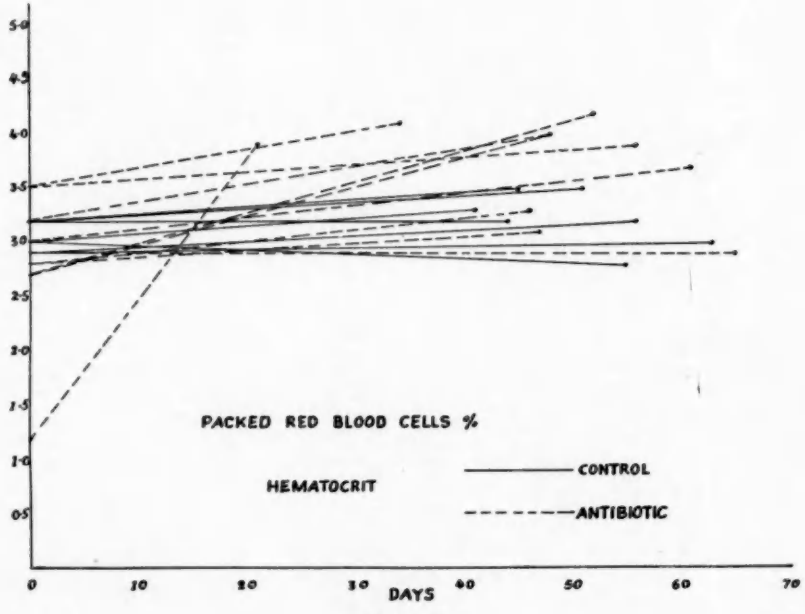

CHART 1

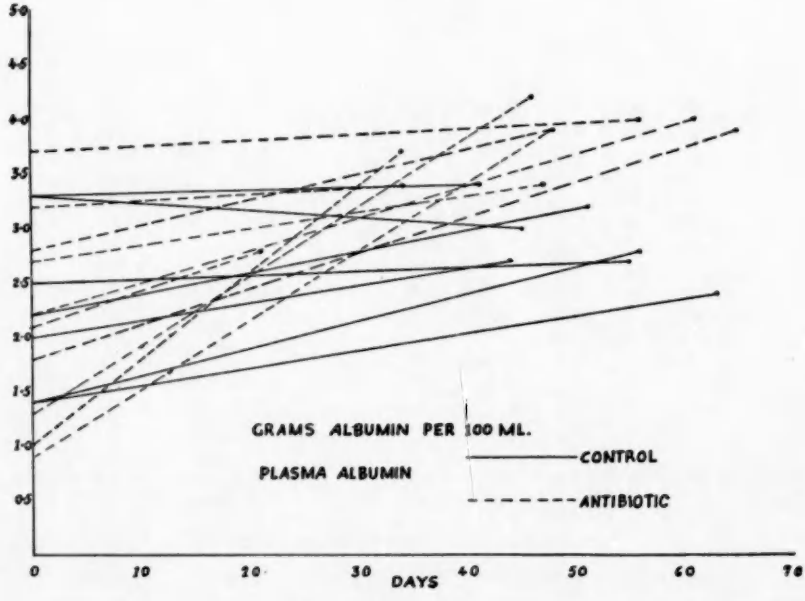

CHART 2

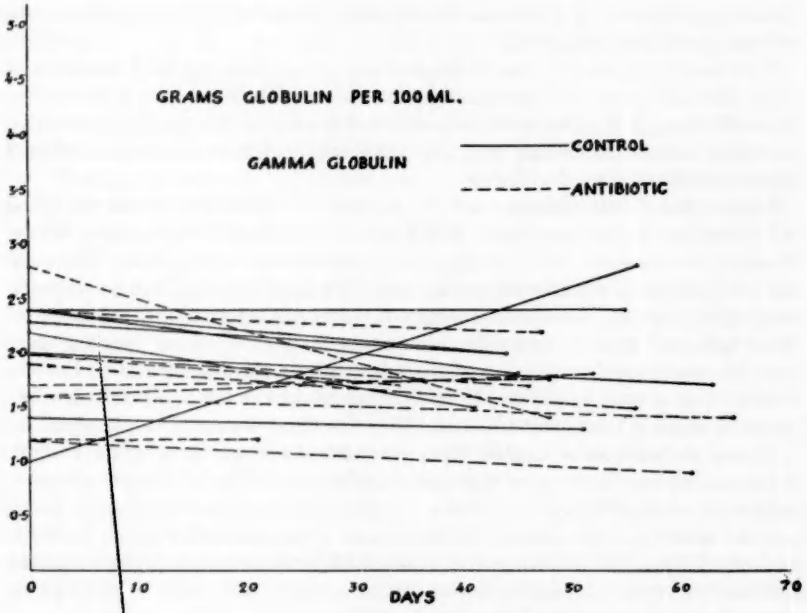

CHART 3

and advanced fatty degeneration of the liver, giving a picture somewhat like the cases of kwashiorkor described in Africa (Gilman, 1945). It is interesting to note that the administration of high protein diet and large amounts of nicotinic acid cleared up the skin lesions but did not bring the hemoglobin or protein values to normal, nor was the fatty condition of the liver brought to normal despite the fact that the second biopsy was made after 55 days of vigorous treatment.

Another group of cases showed marked deficiency of vitamin A which was usually reflected by the presence of Bitot's spots and xerosis which in one case had proceeded to blindness (R8). The association of vitamin A deficiency with kwashiorkor has recently been stressed (Oomen, 1954). Most of our cases, with vitamin A deficiency, showed some evidence of liver damage and vice versa.

Almost all the children had anorexia and apathy and many were bed-ridden. One case, R5, had been unable to walk for two months prior to admission. He was a typical example of kwashiorkor and progress on antibiotic therapy was very rapid. Edema disappeared in a week. In three weeks he was out of bed, walking. After four weeks the liver biopsy was read as showing only slight fatty degeneration and in less than eight weeks it had become normal.

The best response in the control group was made by a child, C1, who had relatively less of protein malnutrition and more of caloric deprivation and would more properly be classified as marasmus. Although he showed the best response to therapy with regard to weight gain, there was only moderate improvement in

the hemoglobin level and the plasma albumin showed a fall, possibly due to the deposition of tissue proteins.

It is interesting to note that there was one case admitted with a diagnosis of "pink disease" which was discussed in the differential diagnosis of kwashiorkor (Trowell *et al.*, 1954). This child, although bed-ridden for two months, responded so well to antibiotic therapy that she was discharged from the hospital after 3 weeks and then followed at home.

Most of the children had a moderate anemia with reduction of the red blood cell count, hemoglobin and hematocrit to about two-thirds of the normal values. However one case with severe anemia, R10, was included in the series. This child had a red cell count of 1.88 million with only 3.6 grams of hemoglobin and a hematocrit of 12 per cent. The first two days in the hospital he was given 350 ml. of blood but after that he was able to maintain an almost normal blood picture until he was taken from the hospital against advice three weeks later. Despite the fact that anemic cases have been reported to show a fall in albumin level at the early stage of treatment (Adams, 1954) this child showed a rise in albumin.

Among the three cases treated with conventional therapy that did not recover there was only one factor in common, namely the very low level of serum albumin. On this basis case C3 may have been more severe than any in the antibiotic group but the other two are comparable with cases given antibiotic which have recovered (R3 and R5). Other workers have had fatal cases and deaths occurring after several weeks of hospitalization (Adams, 1954; Walt *et al.*, 1950) and this matter has been analysed (Trowell *et al.*, 1954).

Although in some cases infection or infestation may have precipitated the disease we do not believe that such factors played much of a part in the development of hypoproteinemia in our cases. There were no instances of hook-worm infestation. Contrasting with the situation in other parts of India there were only three cases of ascariasis. The absence of malaria from Bombay in recent years makes it unlikely that this was a contributory factor and this contrasts with the situation in Africa. On the other hand the dietary habits here may contribute to the clinical picture of the eighteen individuals in the study, the great majority came from homes that were strictly vegetarian, of the Hindu type, so that meat, fish and eggs were often absent from the diet of the mother and child. C1 was Mohammedan but it has been pointed out that he had marasmus rather than hypoproteinemia. C2-R2 received meat once a week and fish twice a week according to the history. Cases C5 and C10 came from families without objection to mixed diet but information was not available on what had been taken. Since vegetarianism influences the plasma protein pattern (Satoskar and Lewis, 1954 a) it may have influenced the protein malnutrition of cases which were studied.

COMPARISON OF RESULTS WITH THOSE OF OTHERS

It is difficult to compare the results of treatment in our series with those which have been published by others because of many differences in the type and uniformity of cases as well as some differences in the laboratory methods used for the determination of protein fractions. Furthermore we were unable to utilise

plasma or protein hydrolysate for intravenous injection and did not have available diets with more than 55 grams of protein including the supplementary casein hydrolysate. Transfusions were given to only two cases and then because of severe anemia. The maximum dietary allowance of 55 grams is much less than the 100 to 120 grams of protein used by Dean and co-workers. Apparently with small doses of antibiotic and 55 grams of protein or less we were able to raise the level of serum albumin as successfully as others who had greater amounts of protein in their hospital diet. Those patients in our series receiving the dietary protein allowance of 55 grams and no antibiotic did not fare well especially in regard to the rise in serum albumin level.

Inspection of Table 5 will show that in our series treated without antibiotic, and in all other series which we have been able to find in the literature there was a tendency for the serum globulin level to rise during treatment and the final level in many cases was higher than normal. On the other hand there was practically no change in the average globulin level of our antibiotic treated patients and inspection of the individual cases will show that in some cases there was a fall in the total globulin level when this was abnormally high before therapy. It may be argued that the rise in gamma globulin which often occurs in kwashiorkor and persists despite treatment is the result of some infection. If so, then this infection, whatever it is, seems to be controlled by the administration of 25 mg. of chlortetracycline or oxytetracycline. In this regard it is worthy of note that among the antibiotic treated cases, it was rarely found necessary to give sulfa drugs for the control of diarrhea. The reduction of plasma globulin in cases of kwashiorkor treated with antibiotics is consistent with the results obtained by Satoskar and Lewis (1954 a and 1955) with similar doses of broad spectrum antibiotics in normal tropical residents. It is possible that the syndrome of kwashiorkor represents an extreme degree of malnutrition which is present to a much smaller extent in almost every person resident in tropical areas who eats the diet which is either necessary or customary in such places.

Another interesting feature of Table 5 is the resemblance between our results without antibiotic and the reports of others as far as the level of hemoglobin is concerned. The rise during treatment reported from Coonoor (Patwardhan,

TABLE 5

Comparison of the results of treatment in our series with other series

Reference	Number of Cases	Duration of Therapy, Weeks	Hemoglobin		Albumin		Total Globulin	
			Before	After	Before	After	Before	After
			gm. %	gm. %	gm. %	gm. %	gm. %	gm. %
This series no antibiotic	7	7	8.9	9.6	2.30	2.89	3.40	3.55
This series with antibiotic	10	7	8.2	11.0	2.18	3.72	3.38	3.02
Patwardhan (India, 1954)	38	4	9.4	9.7	2.35	3.57	2.57	3.47
Adams (Africa, 1954)	8	11	9.5	11.5	1.93	3.88	2.23	2.92
Anderson et al. (Africa, 1951)	6	3	—	—	2.01	3.59	2.12	2.53
Dean et al. (Africa, 1953)	8	4	—	—	1.51	3.50	2.44	3.50

1954) was 0.3 gram, from Africa (Adams, 1954) 2.0 grams and in our series without antibiotic 0.4 gram. But when we added antibiotic the average rise was 3.0 grams. It has already been pointed out that the series without antibiotic had more rigorous antianemic treatment so that this difference is real. Some of us had previously noted (Bhagat and Wagle, 1955) that hemoglobin levels of children with anemia in India frequently failed to come to normal unless a course of antibiotic was given.

Finally it should be noted that in the series summarized in Table 5 we have omitted mention of fatal cases. This point has not been emphasized because the size of the series is small. But it is noteworthy that all of the cases which were treated with small doses of antibiotic recovered and that in the series not given antibiotic there were three deaths relatively late in the period of hospitalization. If the administration of small doses of antibiotic can prevent such accidents this would be sufficient reason for their use in the treatment of kwashiorkor.

EFFECT OF ANTIBIOTICS IN NUTRITIONAL DEFICIENCY

Most workers who have tested the effect of antibiotics upon nitrogen or protein metabolism in the human have used the same dose of antibiotic which is employed for the control of acute infectious diseases (Stoewens 1951; and Kaye *et al.*, 1954). We note a recent report in which one hundred normal young adults were given 250 mg. chlortetracycline for seven weeks and manifested increased weight gain (Haight and Pierce, 1954). We are also aware of unpublished work of a few others who have used 50 to 100 mg. of antibiotic daily. And there has been one experiment in which chlortetracycline was given in doses of 50 mg. daily over a period of almost a year (Scrimshaw and Guzman, 1953). They found that in a group of individuals subsisting on a diet low in animal protein there was an increase in appetite when aureomycin was given in this dosage. Furthermore there was an increase in height and weight but this was below the level of statistical significance in the experiment that was carried out.

Of course there are potential dangers to the long continued administration of antibiotics, even in small doses. If no direct harm to the individual is produced by the antibiotic, there is always the possibility that bacteria may become resistant to the antibiotic used as a food supplement and that this might interfere with treatment of disease caused by such microorganisms. For these reasons we recommend that the problem of antibiotic supplementation of diets low in animal protein be studied on a larger scale.

MECHANISM OF ACTION OF ANTIBIOTICS IN MALNUTRITION

The bulk of evidence from animal experiments points to an effect of small doses of antibiotic on intestinal microflora as the means by which nutrition can be effected (Stokstad, 1954; and Jukes *et al.*, 1954). It is well known that certain bacteria synthesize vitamins and now it has been suggested that B. coli may aid nutrition through its ability to furnish protein of high biological value (Robert, 1954). Others have suggeste that certain bacteria may used up significant amounts of vitamins and if these bacteria are suppressed the nutritional

state may improve (Nutrition Reviews, 1954). Then there is always the possibility that certain bacteria may cause either acute or chronic intestinal disturbances which might be involved in the etiology of kwashiorkor (Gopal and Ramalingaswami, 1954).

It has occurred to us that the name of the disease kwashiorkor refers to the fact that the disease arises in the child that has been weaned (Trowell *et al.*, 1954) and it has recently been shown that the withdrawal of breast milk has a profound effect on the intestinal microflora causing a great decrease in the percentage of gram positive organisms and a noticeable increase in the pH of the intestinal tract (Barbero *et al.*, 1952). It is possible that one of the factors which may operate to precipitate kwashiorkor is change in the bacterial flora of the gastrointestinal tract at the time of weaning. If this hypothesis were correct it suggests that small doses of antibiotic may affect the intestinal flora of children especially after weaning.

SUMMARY

Ten children with protein malnutrition, including several cases of kwashiorkor, were treated with 25 mg. chlortetracycline or oxytetracycline daily for two months. The clinical course of these children was compared with the response of a similar series of cases treated according to conventional methods only. The cases receiving antibiotic in these small doses showed a more rapid and more extensive rise in the blood hemoglobin and plasma albumin levels. A further rise in the average plasma globulin levels did not occur and in the children given antibiotic, when the level was above normal, it declined during the period of therapy. The rate of recovery in the antibiotic treated cases seemed to be more rapid and there were no deaths despite the fact that several of the cases had very low plasma albumin levels and marked fatty infiltration of the liver. The practical and theoretical aspects of the use of small doses of antibiotic as nutritional supplements in the treatment of protein malnutrition are discussed.

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RO 2-1160, A NEW DRUG FOR THE TREATMENT OF AMEBIASIS

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Ro 2-1160,[1] a new pentavalent aromatic stibonic acid (2-carboxymethyl-mercapto-benzine stibonic acid) has been found to be significantly active in curing experimentally induced *E. histolytica* infections in the rat, well tolerated by dogs, and relatively non-toxic in small animals (Schnitzer, 1954; Schnitzer *et al.*, 1951). The present report deals with the results of trials with this agent in human colonic amebiasis.

MATERIALS AND METHODS

The patients were treated and studied at the Tropical Disease Diagnostic Clinic (Lower East Side Health Center, New York City Health Department). The diagnosis of amebiasis was established by the demonstration of cysts and/or trophozoites of *E. histolytica* in casually passed or post-cathartic fecal specimens (direct smear and zinc sulphate centrifugal flotation). After therapy, stool specimens were usually examined at least at tri-weekly intervals. One or more post-cathartic specimens were examined in most cases, usually one month after therapy and subsequently at 1–3 month intervals up to 12 months, in so far as practical. Oral tablets of Ro 2-1160 were administered to a total of 195 patients with colonic amebiasis of varying severity (asymptomatic to moderately severe). These cases were divided into 2 groups: Group A)—an earlier series of 159 patients in which 25 mg./kg. of drug was given daily for 5 successive days: Group B)—a later series of 36 patients in which 25 mg./kg. of drug was given daily for 10 successive days. Ninety-six per cent of all patients were Puerto Ricans of both sexes and their ages varied from 2 to 60.

RESULTS

A) *Parasitological response.* 1. Of the total series of 195 patients comprising Groups A and B, 150 (77%) became and remained stool-negative following treatment (Table 1). In Group A, 116 patients out of 159 (73%) became and remained stool negative (Table 2), whereas in Group B (Table 3) the stool conversion rate was 94 per cent (34 out of 36). In one of the two failures in the latter group an abnormally low dosage of 2.9 mg./kg. was unintentionally employed. In the second patient a positive stool was discovered at the end of the third month after 2 casual and 2 post-cathartic stool specimens examined on different occasions had been found negative.

2. The vast majority of the patients in this series harbored other parasites in

[1] The supplies of Ro 2-1160 used in this study were provided by Dr. R. J. Floody, Hoffmann-La Roche, Inc.

TABLE 1

Results of treatment (total group)

Total Number of Days Followed	Number of Cases	
	Negative	Positive
1–10	10	1
11–20	4	2
21–30	6	2
31–60	22	10
61–90	19	6
91–120	23	4
121–180	35	6
181–360	31	14
Total	150	45
Per cent	77	23

TABLE 2

Results of treatment (group A)

Total Number of Days Followed	Number of Cases	
	Negative	Positive
1–10	10	1
11–20	2	2
21–30	6	2
31–60	18	9
61–90	14	6
91–120	15	3
121–180	20	6
181–360	31	14
Total	116	43
Per cent	73	27

TABLE 3

Results of Treatment (Group B)

Total Number of Days Followed	Number of Cases	
	Negative	Positive
1–10	0	0
11–20	2	0
31–30	0	0
31–60	4	1
61–90	5	0
91–120	8	1
121–180	15	0
181–360	0	0
Total	34	2
Per cent	94	6

addition to *E. histolytica*. *Ascaris*, *Hookworm* and *S. mansoni* did not appear to be affected by the drug, whereas the effect of Ro 2-1160 against *E. coli* was of the same order as against *E. histolytica*.

B) *Clinical response*. Most of the patients exhibited either few or no symptoms. Evaluation of cure by symptomatology was not attempted. However, in those patients with clinical evidence of moderately severe disease (16 cases) treatment with Ro 2-1160 produced rapid clinical improvement. Twelve of these patients were in Group A and 4 were in Group B. The one failure in the moderately severe category occurred in Group A.

C) *Toxicity*. Except for one patient in Group A who complained of slight abdominal discomfort and nausea which did not interrupt treatment, all instances of intolerance were confined to Group B. Symptoms occurred in 6 out of 36 patients in this group and consisted of nausea and vomiting, and in one individual diarrhea as well. In 4 out of the 6 patients who exhibited drug intolerance symptoms began after the first few doses and necessitated discontinuance of treatment. An additional 11 hospitalized patients not followed after treatment consisting of the administration of 25 mg./kg. of drug for 10 consecutive days showed no evidence of hematologic, urinary or hepatic toxicity during or after treatment. One individual in this group had diarrhea during the last four days of treatment and another, a psychotic patient vomited on the fifth, eighth and tenth days of treatment.

DISCUSSION

In interpreting the results of this kind of study the type and adequacy of follow-up is naturally a cardinal point. As can be seen from Table 4 in the majority of patients treated in this series, specifically those followed 31 days or longer, an average of at least 3 stools per case were examined after treatment, 25 per cent of specimens being post-cathartic. If one eliminates from the indicated total of successful stool conversions all individuals followed less than 31 days, the figure for the apparent cure-rate is of course lower, but only by a slight amount. One may arrive at a slightly higher cure rate, on the other hand, by discounting all stool findings recorded later than 6 months after treatment, in

TABLE 4

Adequacy of stool follow-up in negative cases

Number of Days Followed	Number of Cases	Total Number of Stools		Stools per Case (Approx.)	Per Cent Purged
		Casual	Purged		
1–10	10	9	1	1	10
11–20	4	8	1	2	11
21–30	6	11	1	2	8
31–60	22	45	19	3	30
61–90	19	39	18	3	32
91–120	23	53	37	4	41
121–180	35	112	72	5	39
181–360	31	110	47	5	30

effect eliminating new positives. It is possible that by such a method of evaluation many of the cases of reinfection, rather than true failures, are being eliminated. Such reinfection might take several months to involve a particular family member if the whole family had been treated at one time.

The rate of stool conversion may be stated as a range by means of these two methods of evaluation. For the total group the range was 74 to 82 per cent. For Group A it was 70 to 79 per cent; for Group B it was 92–94 per cent.

The better results achieved by the 10-day treatment schedule as compared with the 5-day schedule are statistically significant although the number of cases in the former group is small. Unfortunately, the incidence of toxicity with the longer treatment schedule—essentially nausea and vomiting—was 17 per cent, and this necessitated discontinuance of treatment in 4 out of 6 cases. The actual significance of these findings remains in doubt because the toxic symptoms in the above mentioned 4 cases began within the first 2 to 3 days of treatment. In any event, the above results suggest that an intermediate treatment schedule (7 to 8 days) may be clinically acceptable.

CONCLUSIONS

A new pentavalent aromatic stibonic acid, Ro 2-1160, was administered to 195 patients with asymptomatic to moderately severe amebiasis in doses of 25 mg./kg. per day. One group of 159 patients received the drug for 5 successive days. The stool conversion rate was 73 per cent. The other 36 patients received the drug for 10 days with stool conversion of 94 per cent. The over-all conversion rate for 195 patients was 77 per cent. Toxicity consisting of nausea and vomiting and/or diarrhea occurred in 17 per cent of patients treated for 10 days but was negligible in the 5 day treatment group. In view of the superior therapeutic efficacy observed following 10 days of treatment compared to 5, the former is recommended as the optimum treatment schedule with the reservation that if nausea and vomiting occur after 5 days the drug be discontinued in the presence of such symptoms.

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THE USE OF GLAUCARUBIN (A CRYSTALLINE GLYCOSIDE ISOLATED FROM *SIMAROUBA GLAUCA*) IN THE TREATMENT OF HUMAN COLONIC AMEBIASIS

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Glaucarubin (Merck) is a crystalline glycoside isolated from *Simarouba glauca*. The activity of this agent in vitro against *Entamoeba histolytica* and in experimental animal amebiasis as well as its chemistry and relative toxicity have been reported by Cuckler and Smith (1949), (1950); Phillips (1951); and Taylor and Greenberg (1952). In brief it was found that Glaucarubin possessed sufficient activity and safety in therapeutic amounts to warrant further clinical evaluation.

Trials in human amebiasis have been carried out in Mexico, England, and South Africa by Del Pozo (1955), Woodruff (1955, personal communication) and Elsdon-Dew (1955, personal communication) respectively. Del Pozo (1955) found Glaucarubin as active or more so than currently available amebicides, in a series of 87 cases of chronic amebiasis including 9 patients with "amebic dysentery". The daily dose varied from 20 to 280 mg. and the duration of treatment from 5 to 32 consecutive days. No toxicity was reported and of 54 patients followed from 1 to 13 months 8 failures were recorded. The activity of Glaucarubin in amebiasis with more uniform schedules of treatment is reported by Woodruff (1955) as follows: "53 cases of intestinal amebiasis were treated with Glaucarubin in dosages of 100 to 300 mg. daily during 10 days. Relapse did occur in 20 to 30 per cent of cases treated for 10 days with 200 mg. or more of the drug; the drug was remarkably free from side effects". Elsdon-Dew (1955) and his associates found Glaucarubin moderately effective in acute amebic dysentery. They treated 49 patients for 10 days with 200 mg. of drug daily and noted no toxic effects. The results in their acute clinical material were inferior to other agents tested, notably antimicrobial substances of wide spectral activity. The present study was undertaken to extend these previously reported trials and to define if possible a practical and optimum treatment schedule.

MATERIALS AND METHODS

The patients, mainly Puerto Ricans of both sexes ranging in age from 2 to 42 years and in weight from 15 to more than 50 kg., were in attendance at the Tropical Disease Diagnostic Clinic (Lower East Side Health Center, New York City Health Department). The diagnosis of amebiasis was established by the demonstration of cysts and/or trophozoites of *E. histolytica* in casually passed or post-cathartic fecal specimens (direct smear and zinc sulfate centrifugal

flotation). The drug was administered in tablets by mouth in doses varying from 1 to 5 mg./kg. per day for 5 days and for 10 days to a total of 113 patients with asymptomatic to moderate colonic amebiasis.

RESULTS

Eighty-five patients were considered to have been adequately followed after treatment and the results of their treatment are the basis for the therapeutic evaluation of the drug under study. However, tolerability and toxicity observations were made on 113 individuals treated.

Initially, 26 patients were treated with 1 to 5 mg./kg. of Glaucarubin per day for 5 days. In the follow-up period after treatment, which averaged 169 days during which 3.5 stools per patient were examined, it was found that treatment had failed in 15, or 58 per cent, of the patients treated and accordingly the 5 day schedule was abandoned as unsatisfactory.

Subsequently 59 patients were treated for 10 days with Glaucarubin as follows: 18 patients received less than 5 mg./kg. and 41 received 5 mg./kg. or more per day for 10 days. The maximum daily dose was 300 mg. The failure rates were 38 per cent and 27 per cent respectively for the lower and higher dosage. An average of 2.6 stools per patient were examined during the mean post-treatment observation period of 82 days. Eleven patients were followed less than 60 days and for the remainder the follow-up period was from 60 to 200 days. Glaucarubin had no therapeutic effect on the helminths (*ascaris*, hookworm, *trichuris*, *S. mansoni*) which were also present in some patients treated for amebiasis. On the other hand, amebae other than *E. histolytica* apparently were affected to the same extent as the latter, disappearing from approximately 70 per cent of the patients treated for 10 days with 5 mg./kg. Glaucarubin per day.

TOXICITY

The drug was well tolerated by the patients. In 2 individuals of 113 who received Glaucarubin for 5 or 10 days, vomiting necessitated discontinuance of the drug. In one patient who was receiving 300 mg. of Glaucarubin daily the white count was noted to be 2,700 on the fourth day of therapy at which time the drug was discontinued. Six days afterwards it had risen to 8,000 (pre-treatment wbc = 7,600). In the remainder of the patients there was no evidence of clinical toxicity or disturbance in hematologic, hepatic or renal function.

SUMMARY

Glaucarubin, a crystalline glycoside isolated from *Simarouba glauca* and found to have amebicidal properties in vitro and in experimental animal amebiasis, was evaluated in the treatment of the human infection. Cure rates in the order of 70 per cent were observed following treatment by mouth for 10 days with daily doses of 5 mg./kg. (maximum daily dose 300 mg.). With the exception of vomiting in 2 patients and a transitory decrease in leucocyte count in another, the drug was well tolerated in 113 patients.

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INFLUENCE OF THE AMINO NUCLEOSIDE OF PUROMYCIN ON THE COURSE AND PATHOLOGY OF TRYPANOSOME INFECTIONS IN RABBITS AND MICE

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Recently the antibiotic Puromycin has been reported to be highly active against pathogenic trypanosomes in laboratory animals (Porter *et al.*, 1952; Hewitt *et al.*, 1953; Tobie, 1954) and against human sleeping sickness caused by *Trypanosoma gambiense* (Trincão *et al.*, 1955). Studies on the influence of Puromycin and its amino nucleoside on the metabolism of *T. equiperdum* indicate that these compounds interfere with the purine metabolism rather than the carbohydrate metabolism (Agosin and von Brand, 1953) and with the synthesis of nucleic acids or nucleoproteins (Hewitt *et al.*, 1954). There are also indications that Puromycin may be changed rapidly to a more potent compound *in vivo* (Agosin and von Brand, 1953).

Since the amino nucleoside is an active component of Puromycin it seemed important to compare its activity against several species of trypanosomes. In this report will be presented the trypanocidal activity of the amino nucleoside of Puromycin against four species of trypanosomes, and preliminary studies on the pathology produced when infections of *T. gambiense* in mice are prolonged by treatment with subcurative doses of the amino nucleoside.

MATERIALS AND METHODS

The following species were selected as representatives of various groups of trypanosomes; *Trypanosoma gambiense* and *T. rhodesiense* of the *brucei* group; *T. congolense* of the *congolense* group; and *T. equinum* of the *evansi* group. The amino nucleoside of Puromycin was obtained from Lederle Laboratories. The LD 50 value of the amino nucleoside when administered intraperitoneally to mice is 325 mg. per kg. (Dr. R. I. Hewitt, personal communication). Concentrations used were in proportion to those used for Puromycin as based on the LD 50 values of the two compounds.

Swiss mice of the NIH general purpose strain and New Zealand white rabbits were used as host animals. Mice, usually females, were inoculated intraperitoneally as previously described (Tobie, 1954), and the inoculum was varied as given in the tables. In most experiments mice were treated intraperitoneally with a single dose of the desired amount of the amino nucleoside dissolved in 0.2 ml. of saline. Negative survivors were observed for relapse for 60 days following treatment. Rabbits weighing approximately 1 kilogram were inoculated in-

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traperitoneally with approximately 1,000,000 trypanosomes and treated intraperitoneally daily until the desired total dose of drug had been given. Animals considered cured were observed for relapse for 7 to 10 months following cessation of treatment.

When an attempt was made to produce resistance to the amino nucleoside two *in vivo* methods were used. In one, male mice were retreated with the same dose after each relapse until death seemed imminent. In the other, female mice were treated at the height of infection, and when relapse occurred, transfer was made by subinoculation to fresh mice and the procedure repeated.

In each experiment, regardless of host, the course of infection before treatment, the effect of the compound on parasitemia, and the relapse when it occurred were followed by microscopic examination of fresh drops of blood. Parasitemia was evaluated as in previous studies (Tobie and von Brand, 1953). In addition the weight of each rabbit was followed since weight loss accompanied the progress of the disease and was therefore an indication of relapse.

The tissues for histologic study were fixed in a 10 per cent aqueous solution of buffered formalin (pH 7.0). Routine paraffin sections were stained with hematoxylin azure eosin. Certain sections were also stained by the periodic acid-Schiff procedure and for calcium, collagen, hemosiderin, and myelin. Frozen sections of the heart, liver, and kidney were stained for fat with Oil red O (Lillie, 1954).

EXPERIMENTAL DATA

Effect of the amino nucleoside of Puromycin on trypanosome infections in mice

The data of a large number of experiments on the trypanocidal effect of the amino nucleoside of Puromycin on *Trypanosoma gambiense*, *T. rhodesiense*, *T. congolense*, and *T. equinum* infections in mice are summarized in Table I. When administered as a single dose, 80 mg. per kg. was found to be very effective against *T. gambiense*, and therefore this amount of the drug was used in most tests. Control animals began dying at 3.5 days when given the usual inoculum of 10,000 trypanosomes, which gave a chance for successful treatment of mice on days 1 and 2 and of a group with high parasitemia on day 3. In 4 experiments in which 80 mg. per kg. of the drug was administered as a single dose, no mice relapsed, regardless of day of treatment, but in others a few did relapse. The drug had less activity against *T. equinum* and *T. rhodesiense* when treatment was delayed 2, 3, and 4 days. It was least effective against *T. congolense*. Since the *T. congolense* strain used is less virulent to white mice than the strains of the other species, an inoculum of 500,000 trypanosomes was used as well as the usual 10,000. When this larger inoculum was given no mice survived except in groups treated on day 4 and 7. Regardless of species, the blood stream was cleared for a period of several days following treatment before relapse occurred (Table 1).

Table 2 gives the effect of inocula of different size on cure rate of groups of mice infected with *T. gambiense* and treated with a single dose of 80 mg. per kg. on one of the three days following inoculation. It is evident that both a larger inoculum and delayed treatment reduced the chance of cure (Table 2).

TABLE 1

The effect of the amino nucleoside of Puromycin on trypanosome infections in mice

Trypanosome species	*congolense*	*congolense*	*equinum*	*equinum*	*gambiense*	*gambiense*	*gambiense*‡	*gambiense*‡	*rhodesiense*
No. of trypanosomes inoculated	10,000	500,000	100,000	100,000	10,000	10,000	10,000	10,000	10,000
Controls									
Average survival in days (range)	7.9(7.0–9.0)§	6.8(6.5–7.5)§	4.9(3.5–6.0)		4.7(3.5–6.0)		4.7(3.5–7.5)		5.4(4.5–8.0)
Total mg. of drug per kg. body weight/ No. doses	80 mg./4	80 mg./1	40 mg./1	80 mg./1	40 mg./1	80 mg./1	80 mg./1	160 mg./1	80 mg./1
Rx started day 1									
Negative survivors	7	0	10	9(1)*	4	31(4)*		10	10
No. relapsed/No. inoculated	3/10	10/10	0/10	0/10	6/10	0/35		0/10	10/20
Average suppression in days (range)	9.0	6.6(6.0–7.0)			9.2(7.0–13.0)				10.2(8.0–15.0)
Rx started day 2									
Negative survivors	7	0	5(4)*	8	5	31(3)*		10	9
No. relapsed/No. inoculated	3/10	10/10	1/10	2/10	5/10	1/35		0/10	11/20
Average suppression in days (range)	8.0	6.2(5.0–7.0)	17.0	18.5(17.0–20.0)	9.6(7.0–12.0)	7.0			9.5(6.0–15.0)
Rx started day 3									
Negative survivors	5	0	6	8	3	39(4)*	5	10	3
No. relapsed/No. inoculated	5/10	10/10	4/10	2/10	7/10	2/45	5/10	0/10	17/20
Average suppression in days (range)	8.6(8.0–9.0)	7.3(5.0–14.0)	18.0(15.0–19.0)	23.0	8.0(6.0–11.0)	9.5(8.0–11.0)	36.4(14.0–45.0)		8.4(7.0–11.0)
Rx started day 4									
Negative survivors	4	3	2	4(3)†	0	1(3)†			0
No. relapsed/No. inoculated	6/10	7/10	8/10	3/10	10/10	1/5			20/20
Average suppression in days (range)	6.8(6.0–8.0)	8.6(7.0–9.0)	19.3(15.0–33.0)	22.7(15.0–29.0)	6.8(6.0–9.0)	10.0			8.6(7.0–18.0)
Rx day 7									
Negative survivors		1(7)†							
No. relapsed/No. inoculated		2/3							
Average suppression in days (range)		5.0(4.0–6.0)							

Figures in parentheses indicate number of deaths.

* Death not due to trypanosomiasis.

† Died before treatment could be given or take effect.

‡ Male animals; all others females.

§ Average for 5 of 10 animals which died. Other 5 finally had to be killed.

TABLE 2

The effect of the size of the inoculum on cure of mice infected with Trypanosoma gambiense *when treated with a single dose of 80 mg. per kg. of the amino nucleoside of Puromycin*

	Number of Trypanosomes Inoculated			
	1,000	10,000	100,000	1,000,000
Controls				
Average survival in days	4.6	4.3	3.7	2.9
Range	4.5–5.5	3.5–4.5	3.0–5.0	2.5–3.5
Rx day 1 (parasitemia)‡	(−)	(p)	(+)	(+)
Negative survivors	7 (3)*	10	10	10
Number relapsed/Number inoculated	0/10	0/10	0/10	0/10
Rx day 2 (parasitemia)	(p)	(+)	(++)	(+++)
Negative survivors	6	8 (1)*	8	5
Number relapsed/Number inoculated	4/10	1/10	2/10	5/10
Rx day 3 (parasitemia)	(+)	(+++)	(+++ to ++++)	
Negative survivors	9	7	0	
Number relapsed/Number inoculated	0/10 (1)*	3/10	3/10 (7)†	

Figures in parentheses indicate number of deaths.

* Cause of death not determined.

† Mice with parasitemia of ++++ died before drug could be effective.

‡ Evaluations at time of treatment according to Tobie and von Brand 1953.

TABLE 3

Schedule of treatments in attempt to produce resistance to the amino nucleoside of Puromycin when individual male mice were treated with 80 mg. per kg. following succeeding relapses

Mouse	Treatment Number								
	1	2	3	4	5	6	7	8	9
A2									
Day of treatment*	3	58	87	114	128				
Days between treatments	55	29	27	14					
A4†									
Day of treatment	3	76	87	98	111	122	133		
Days between treatments	73	11	11	13	11	11			
A6†									
Day of treatment	3	45	74	98	115				
Days between treatments	42	29	24	17					
A8									
Day of treatment	3	52	87	106	122	133			
Days between treatments	49	35	19	16	11				
B1 (subinoculated from A6)†									
Day of treatment	5	35	77	108	140				
Days between treatments	30	42	31	32					
B2 (subinoculated from A4)†									
Day of treatment	3	25	35	44	53	62	72	81	91
Days between treatments	22	10	9	9	9	10	9	10	

* Days after inoculation.

† Tissues saved for histologic study.

TABLE 4

Trypanosoma gambiense *in rabbits treated with the amino nucleoside of Puromycin*

Rabbit No.	Parasitemia Prior to (Death) and (Treatment)	Treatment		Death, Cure, or Relapse/Days
		Day begun	Regimen, daily dose/no. doses	
		Controls		
1‡	+++ (death)			Dying, sacrificed/63
5	?			Died/62
7	p (death)			Died/46
9	+ (death)		*	Died/43
15‡	++ (death)			Sacrificed/50
16	− (death)			Died/55
18‡	p (death)		*	Died/43
		Treated		
2‡	− (death)	63	40 mg./5	Apparent cure; gangrenous hind legs; sacrificed/172
3	?	63	20 mg./10	Apparent cure/300
8	− (treatment)	55	20 mg./14*	Apparent cure/220
10	+ (treatment)	50	20 mg./13*	Apparent cure/220
13	p (treatment)	55	20 mg./14	Apparent cure/220
12	− (treatment)	55	20 mg./14†	Died/74
6‡	p (death)	36	25 mg./4	Relapsed/67; failed rapidly; sacrificed/95
11‡	p (treatment)	52	20 mg./2†	Died/55
17	++ (death)	49	20 mg./1†	Died/50

* Animals given streptomycin and penicillin.

† Died during treatment or immediately following treatment.

‡ Tissues saved for histologic study.

In one experiment where 10 each of male and female mice were treated on day 3, 5 of 10 males relapsed while at least 9 of the 10 females did not relapse. (These data are incorporated in Table 1.) The cause of the death of the one female was undetermined. This may suggest a sex difference in response to the drug.

In an attempt to produce resistance to the amino nucleoside, the following trials were made. Since male mice relapsed when a single dose of 80 mg. per kg. was given, they were treated following succeeding relapses. Data on six mice thus treated are given as representative in Table 3. In these animals, following each treatment, the blood stream was cleared, but the negative periods were shortened. Four mice were sacrificed for histopathologic studies when it seemed certain that they could not survive another relapse. The others eventually died. There was little chance of relapse when female mice were given 80 mg. per kg., but when a single dose of 40 mg. per kg. was used, relapses as well as cures resulted, as shown in Table 1. Therefore, female mice were used for study of treatment effects following relapse and transfer. Six consecutive transfers were made following relapse and 40 mg. per kg. administered at the height of infection

each time. Then the mice of the succeeding four passages were given 80 mg. per kg. Relapses occurred in transfers 7, 8, and 9, but complete cure resulted in transfer 10. In view of these results, it was not felt that true resistance to this drug was produced in *T. gambiense* at the concentrations used.

Effect of the amino nucleoside of Puromycin on Trypanosoma gambiense *in rabbits*

In rabbits, *T. gambiense* produces a disease which progresses slowly and is marked by a low parasitemia in contrast to the acute disease with extremely high parasitemia produced in mice, rats, and guinea pigs.

Parasites were detected in the blood-stream of rabbits the day following inoculation. In general, parasitemia remained low as is shown in Table 4. One to two weeks following inoculation external lesions appeared chiefly around the eyes and on the nose and ears. These rapidly grew worse until several animals were scarcely able to see and eat (Figures 1 and 2). To make sure that these lesions were not due wholly or partially to a secondary infection, penicillin and streptomycin were given to infected control rabbits 9 and 18 and also to rabbits 8 and 10 prior to treatment with the amino nucleoside. These antibiotics did not prolong the life of the controls, and no change was evident in the lesions of the test animals until treatment with the amino nucleoside was begun. Untreated animals died

FIG. 1. Rabbit 10. 50 days after inoculation with *T. gambiense* showing severe lesions on day treatment with the amino nucleoside was begun.

FIG. 2. Rabbit 13. 55 days after inoculation with *T. gambiense* showing severe lesions on day treatment with the amino nucleoside was begun.

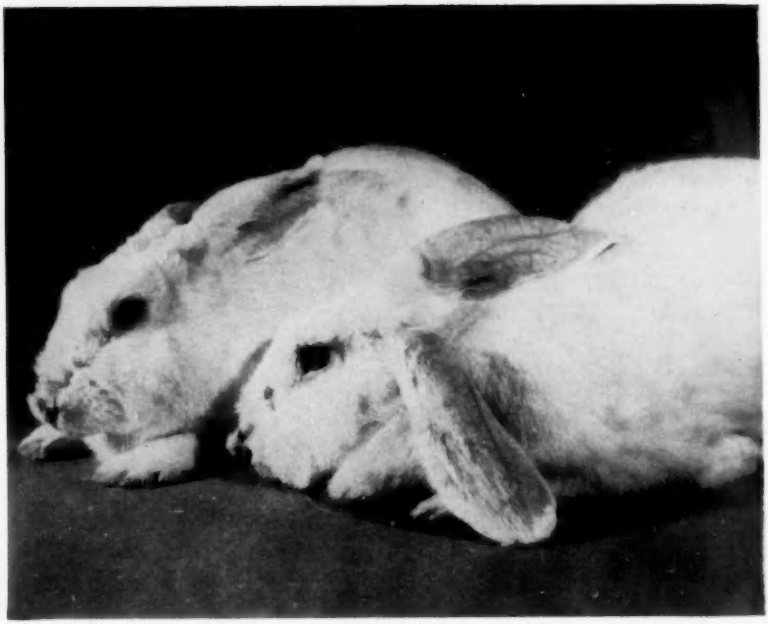


FIG. 3. Rabbits 10 and 13. 7 to 8 months after cessation of treatment. Lesions are healed but deformities of nose due to lesions remain.

between 43 and 63 days. Treatment was started late on the other animals, mostly after day 50.

Two animals died before treatment was completed, and one died immediately following the completion of treatment. A total of 100 mg. per kg. did not cure one rabbit, number 6, but 200, 260, and 280 mg. per kg. resulted in apparent cure of five animals. Table 4 gives complete data concerning treatment, death, cure, and relapse of the various rabbits. Figure 3 shows rabbits 10 and 13 cured, of trypanosomiasis by the amino nucleoside of Puromycin. All cured animals have gained weight steadily since treatment was begun.

Histologic observations on mice and rabbits infected with Trypanosoma gambiense

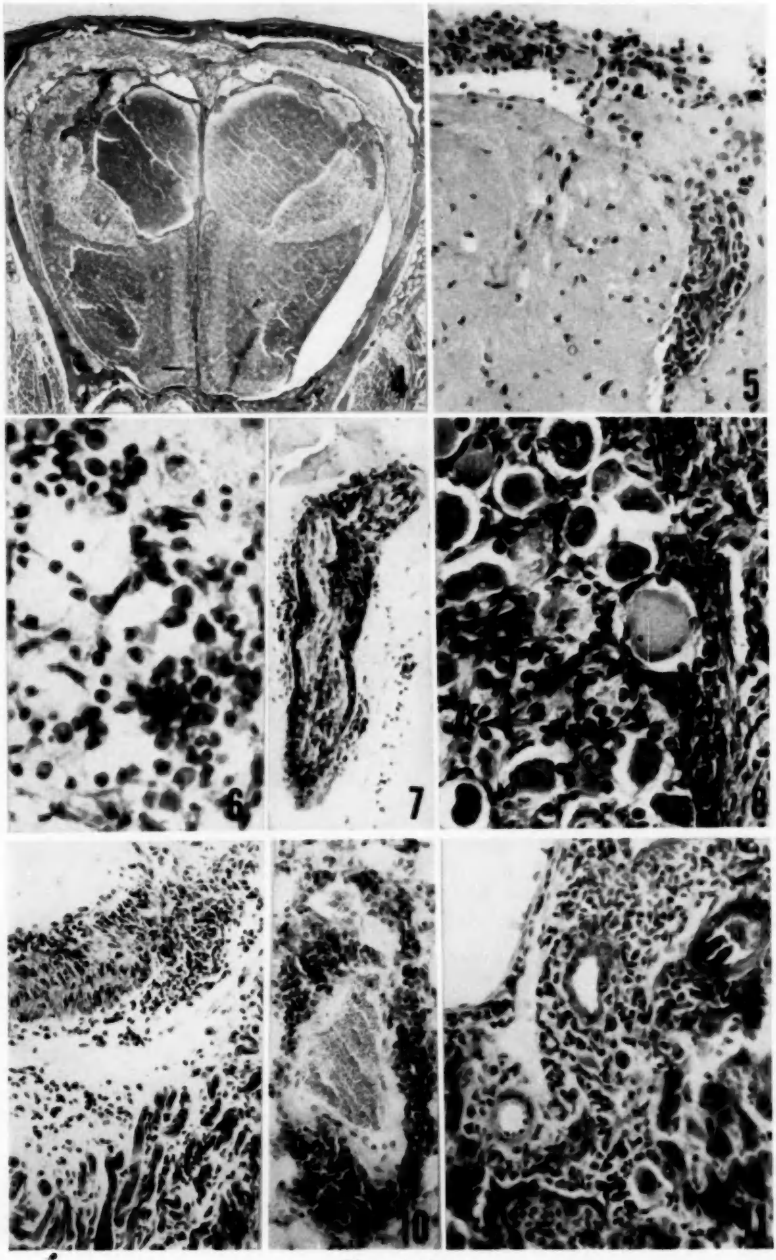
Mice. The organs of four treated mice (A_4, A_6, B_1, and B_2 in Table 3) sacrificed 98 to 147 days after inoculation were studied histologically. The organs included the heart, lungs, liver, spleen, kidneys, and brain. The brains of two mice (B_1 and B_2) were removed from the skull before fixation, while those of the other two (A_4 and A_6) were sectioned with the skull after formalin fixation followed by decalcification in 5 per cent formic acid.

Sections revealed in nearly all organs focal collections of inflammatory cells (Figures 4 to 11); lymphoid cells and plasma cells usually predominated. Russel's fuchsin bodies were seen rarely. The changes were most marked in the meninges and liver. No trypanosomes were identified.

Brain. All the mice showed a dense patchy infiltration of the pia-arachnoid by inflammatory cells, chiefly plasma cells and lymphoid cells (Figures 4, 5, 6). In some areas there were numerous large mononuclear cells grading into fibroblasts and some polymorphonuclear leucocytes. Many plasma cells were abnormal in size, shape, and staining characteristics (Figure 6). There were a few cells that had hyaline eosinophilic cytoplasmic inclusions and resembled the morula cells of Mott. In some areas, inflammatory cells were seen around blood vessels extending from the meninges into the brain substance, and occasional inflammatory cells were noted in the brain tissue adjoining these infiltrations (Figure 5). Sections of the skull disclosed dense accumulations of inflammatory cells extending from the basal meninges through the foramina forming a sheath around nerve trunks and ganglia. There were a few inflammatory cells within nerve trunks and ganglia, and a few ganglion cells were necrotic (Figures 7 and 8).

Sections at the level of the eyes revealed architectural distortion in the rhinencephalon subjacent to the frontal pole of the cerebral hemispheres (Figure 4). Between the hemispheres and beneath the calvarium, there was marked vascular dilation and a dense inflammatory infiltration of the pia-arachnoid (Figure 4). The dura was densely fibrous and thick (Figure 4), focally infiltrated by inflammatory cells, and adherent to the pia-arachnoid; dural thickening may be seen in this area occasionally in uninoculated controls. In one eye of mouse A_6, the iris showed a few inflammatory cells and was focally adherent to the anterior capsule of the lens and to the posterior surface of the cornea.

Liver. Sections revealed large inflammatory foci in many portal areas (Figure 11) and occasional smaller foci in the sinusoids and along the central veins. The



FIGS. 4–11

inflammatory cells were similar to those described in the meninges, but lymphoid cells usually predominated; hematopoietic cells and megakaryocytes were noted occasionally (Figure 11). In the vinicity of the large periportal inflammatory foci, the sinusoids often contained many inflammatory cells, and the liver cells and cords were disorganized, compressed, atrophic, and occasionally necrotic. The Kupffer cells were increased in number and size and occasionally contained phagocytosed red or white blood cells or debris. One mouse (A_6) showed a small subcapsular area of coagulation necrosis and a small amount of lipoid (small droplets) in the liver cells centrolobularly.

Kidneys. Sections disclosed only occasional small collections of inflammatory cells surrounding arteries and lying beneath the renal capsule and pelvic epithelium or in the perirenal fatty tissue.

Lungs. Occasional inflammatory foci were noted around blood vessels and bronchi and in interlobular and interalveolar septa (Figure 10).

Heart. Perivascular inflammatory foci were seen only rarely. One mouse (B_2) showed an inflammatory focus at the root of the aorta with inflammatory cells infiltrating the intima and adventitia of the aorta and the fibrous tissue between the muscle fibers of the adjacent auricle (Figure 9).

Other organs. The spleen showed prominent splenic corpuscles, moderate hematopoietic activity, and a small amount of hemosiderin. In the skeletal musculature, a few perivascular inflammatory foci were noted. No significant changes were seen in the adrenal and bone marrow.

Rabbits. Histologic studies were made on the brains of two treated rabbits (2 and 6 in Table 3) and 4 untreated infected rabbits (1, 11, 15, and 18) as well as of six uninfected rabbits used as controls. No unequivocally significant changes were noted in the brain substance of any of the infected rabbits. However, in comparison with the uninfected controls, the ventricles of the brains of two rabbits

FIG. 4. Cross section at eye level of skull of mouse A_6 treated with the amino nucleoside of Puromycin and killed 142 days after inoculation with *T. gambiense*. Note in upper portion of figure marked thickening and adherence of dura to congested and inflamed pia-arachnoid and architectural distortion of subjacent brain. Hematoxylin azure eosin × 40.

FIG. 5. Section of brain of mouse B_2 treated with the amino nucleoside after relapses and killed 98 days after inoculation with *T. gambiense*. Note cellular infiltration of pia-arachnoid and Virchow-Robin space in cerebral cortex. Hematoxylin azure eosin × 290.

FIG. 6. Higher magnification of portion of pia-arachnoid from Figure 1 showing plasma cells and other types of inflammatory cells. Hematoxylin azure eosin × 465.

FIG. 7. Branch of cranial nerve of mouse A_6 showing inflammatory cells in perineurium and between individual nerve fibers. Hematoxylin azure eosin × 150.

FIG. 8. Gasserian ganglion of mouse A_4 treated with the amino nucleoside following relapses and killed 142 days after inoculation with *T. gambiense* showing necrosis and lesser degenerative changes involving many of the ganglion cells and numerous inflammatory cells infiltrating the ganglion and its capsule. Hematoxylin and eosin × 350.

FIG. 9. Heart of mouse B_2 showing inflammatory cells infiltrating fibrous tissue between muscle fibers of right auricle in lower portion of figure and intima, adventitia, and root of aorta in upper portion of figure. Hematoxylin azure eosin × 150.

FIG. 10. Lung of mouse A_6 showing collar of inflammatory cells around artery. Hematoxylin azure eosin × 200.

FIG. 11. Liver of mouse B_2 showing inflammatory cells infiltrating portal area and adjacent sinusoids. Hematoxylin azure eosin × 240.

(1 and 2) were slightly dilated, and three rabbits (1, 2, and 9) exhibited excessive calcareous deposits in the ventricles or along the ependymal surface. Two uninfected controls showed a few small calcareous deposits in or along the ventricles. Sections of the lung of rabbit 18 (Table 4) revealed an extensive pneumonic consolidation; large mononuclear cells predominated in the alveolar exudate and polymorphonuclear leucocytes in the bronchial exudate. Two elongated degenerating trypanosomes were noted in an alveolus near the pleural surface.

DISCUSSION

In a previous study (Tobie, 1954) it has been shown that Puromycin is highly effective against various trypanosome infections in mice including those due to *T. gambiense* and *T. rhodesiense*. Since encouraging results with Puromycin in human infections have been reported (Trincão *et al.*, 1955) it seems possible that in the future antibiotics may play a role in the treatment of trypanosomiasis. In view of this, the data presented in this report are of interest because they show that part of the Puromycin molecule, the amino nucleoside, is even more active than Puromycin itself. This is especially true against the human parasites *T. gambiense* and *T. rhodesiense* but it is also true concerning *T. equinum*. The compound has also been shown to be active against *T. equiperdum* (Hewitt *et al.*, 1954). The previous study (Tobie, 1954) and the present one have shown that both Puromycin and its amino nucleoside are definitely less active against *T. congolense* than against the other species studied but even against *T. congolense* the amino nucleoside was somewhat more active than the parent compound.

In the past, chemotherapeutic studies on the late stages of infection with African trypanosomes have been handicapped by the lack of a small laboratory animal in which specific lesions of the central nervous system could be produced readily. Such lesions have been produced occasionally in primates (Peruzzi, 1927; Hoeppli and Regendanz, 1930), and there is at least one strain of *T. gambiense* with extremely low virulence which produces central nervous system symptoms in rats (Launoy, 1951), but smaller laboratory animals usually die after a short acute period of infection. The hypothesis has been advanced (Thomas and Breinl, 1905) that a prolonged period of infection is required before lesions develop in the central nervous system. This hypothesis is supported by our study. In this study, by treating infected mice after each of a series of relapses, survival time has been greatly prolonged; this has made it possible to produce in the mouse changes in the central nervous system comparable with those produced by *T. gambiense* in the late stages of infection in man. The possibility that similar lesions may be produced in other laboratory animals by this method is under investigation. The use of such laboratory animals may facilitate chemotherapeutic studies and perhaps hasten the development of more effective therapy of the late stages of *T. gambiense* infection in man.

The changes described in other organs of the mice are essentially similar to those found by other investigators and those found in human infection. The absence of unequivocal lesions in the central nervous system of the rabbits is

not surprising in view of the relatively short duration of the disease in the untreated animals.

SUMMARY

The amino nucleoside of Puromycin was tested for its trypanocidal activity against *Trypanosoma congolense*, *T. equinum*, *T. gambiense*, and *T. rhodesiense* infections in mice and *T. gambiense* in rabbits. Data reported show that the drug was somewhat effective against *T. congolense* only when a small inoculum was used but was highly effective against the other species especially *T. gambiense*. By treating mice following each of a series of relapses it was not possible to produce true resistance to the drug but it was possible, due to the prolonged course of the infection, to produce changes in the central nervous system of the mouse comparable to those produced by trypanosomiasis in man.

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A STUDY OF THE ETIOLOGY OF "ACCLIMATIZATION DIARRHEA" AMONG AMERICANS IN EGYPT[1]

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Diarrheal diseases are common in areas of unsatisfactory sanitation and are frequently experienced by travelers and newly arrived residents in those localities. While acute bacillary dysentery is encountered, the majority of these attacks are of a milder type characterized by diarrhea without much constitutional disturbance. The temperature is slightly elevated, nausea and vomiting are occasionally present, stools are frequent and liquid in consistency but rarely contain blood and mucous. Improvement generally occurs within forty-eight to ninety-six hours. The episodes usually are considered by the lay public to be due to unaccustomed food, water or climate and are spoken of as "acclimatization diarrhea", or are given names such as "Simla trots", "Hongkong dog" and "Delhi belly", varying with the locality in which they occur. While a variety of bacterial, protozoal and helminthic organisms have been suggested as possible etiological agents (Gunn, 1946; Kean and Smillie, 1954; Mackie, Hunter and Worth, 1945), most of the cases fall well within the present concept of shigellosis (Bockus, 1944; Borg, 1946; Bulmer, 1943; Felsen, 1945; Manson-Bahr, 1939; Perry, 1930; Perry and Bensted, 1928; Weil, 1943; Wirts and Tallant, 1944). However, since medical care often is not sought or limited facilities prevent laboratory assistance in establishing a diagnosis, the use of these vague lay terms persists and a distinction continues to be made between these mild diarrheas of uncertain etiology and bacillary dysentery.

"Acclimatization diarrhea", or "Gyppy tummy" as it is more widely known, is a commonplace, seemingly inevitable, illness of recently arrived foreigners in Egypt. Attacks are sudden, numerous and annoying at first but become less frequent and milder with repeated infection. Relatively few studies have been made of the etiology of these mild diarrheal episodes (Clegg, 1941; Perry and Bensted, 1928; Wirts and Tallant, 1944).

In an effort to determine the extent of association between these disorders and the presence of *Shigella* organisms, a study of the etiology of "Gyppy tummy" in Americans in Cairo was conducted over a three year period between 1951 and 1954. If confirmation could be obtained for the postulation that these disorders are, in effect, manifestations of shigellosis, diagnosis and treatment would then be on a rational foundation.

[1] The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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MATERIALS AND METHODS

Rectal swabs and/or fresh stool specimens were obtained on a voluntary basis from American personnel and their dependents, of this unit and of the American Embassy, who were suffering at the time with "Gyppy tummy". Due to the ordinarily mild nature of the episodes many of the cases were self-diagnosed. A few with persistent symptoms were seen by a physician at this unit or by a nurse in charge of the Embassy dispensary. The principal criterion used for the inclusion of a case in the study was the presence of frequent, urgent and watery stools. In view of the manifold diseases with diarrheal manifestations the inherent errors in this type of selection were well recognized. However, it was felt that without clinical examination of each case by a physician, which would have been impossible, more rigid criteria would have been of little value. With the exception of a young child later shown to have had typhoid fever, none of the cases proved to have been anything more than episodes of mild diarrhea.

TABLE 1

Incidence of Shigella *organisms in cases of "Gyppy tummy" and in non-diarrheal disease controls among Americans in Cairo, Egypt*

Year	Number subjects	Cultures			Estimated Cases		
		Total number	Positive Shigella		Total number	Shigella present	
			Number	Per cent		Number	Per cent
1951–2	64	102	29	28.4	90	22	24.6
1952–3	40	72	17	23.6	52	17	32.6
1953–4	66	131	33	25.1	84	30	35.7
Total	170	305	79	25.9	226	69	30.5
Controls 1951–4	—	201	8	3.9	—	—	—

TABLE 2

Shigella *species and serotype distribution in cases of "Gyppy tummy" among Americans in Cairo, Egypt*

Organism	No. Isolations
S. dysenteriae 3–7	5
S. flexneri 2a	15
S. flexneri 3	14
S. flexneri 5	12
S. flexneri 6	7
S. variant type "X"	1
S. boydii 1	1
S. boydii 2	1
S. boydii 3	1
S. boydii 4	3
S. boydii 5	1
S. sonnei	18
Total	79

Each specimen was streaked on a Shigella-Salmonella agar (Difco) and a MacConkey agar (Difco) plate. Tetrathionate broth (Difco) in flasks was inoculated, incubated for 18–24 hours and then subcultured onto S-S and MacConkey agar plates. All plates were incubated for 24 hours at 37°C. and suspicious non lactose-fermenting colonies transferred to triple sugar-iron agar (Difco) slants. These were incubated and examined for reactions typical of *Shigella* or *Salmonella*. Organisms showing such reactions were tested for their biochemical properties. Strains with characteristic biochemical behavior were finally identified by serological methods. Other enteric organisms were identified as far as was feasible.

Examination of stools for protozoa and helminth eggs was performed by the M.I.F. (merthiolate-iodine-formalin) technic of Sapero and Lawless (1953) and by a modification of that technic by Blagg and coworkers (1955). A group of 201 rectal swab or stool specimens from Americans during routine physical examinations or with other diagnoses were cultured according to the method described and served as a control.

RESULTS

On the average a single rectal swab or stool specimen was obtained from each individual case. Replicate swabs or stools were received from a few persistent cases. During the three-year period from August, 1951, to August, 1954, a total of 305 specimens was collected from 170 individuals. It was estimated that these represented 226 separate attacks of diarrhea.

Shigella organisms were recovered from 79 (25.9%) of the 305 cultures. *Shigella* organisms were associated with 69 (30.5%) of the 226 estimated diarrheal episodes. When examined by individual years the results show no appreciable differences in percentages of positive cultures (Table 1). Species and serotype distribution of the *Shigella* organisms recovered are shown in Table 2. No *Salmonella* organisms were found.

Forty-eight of the cultures yielded paracolon organisms; six of the strains were of the Bethesda group. *Proteus* were recovered on 42 occasions. The majority, by biochemical differentiation, were *Proteus rettgeri* and *Proteus morgani*.

It was not possible to culture vomitus or suspected foods for the presence of enterotoxin producing staphylococci. There were no recognized outbreaks of staphylococcal food poisoning among this group of subjects during the study.

Entamoeba histolytica trophozoites or cysts were found in 10 (4.1%) of the 239 stool specimens examined for protozoa and helminth eggs. *Dientamoeba fragilis* was noted in 5 (2.0%), *Giardia lamblia* in 7 (2.9%) and *Iodamoeba bütschlii* in 1 (0.4%). No helminth eggs or potentially pathogenic protozoa were observed in the remaining 216 (90.3%) stools.

Shigella organisms were recovered from 8 (3.9%) of the 201 control cultures. Parasitological data on these specimens were not available.

DISCUSSION

From the results presented here it is evident that a significant percentage of the diarrheal episodes referred to as "Gyppy tummy" in the group under study

were associated with infection with *Shigella* organisms. It is felt that these results probably represent a minimum percentage in view of the difficulty often encountered in recovering *Shigella* from a single stool or rectal swab culture and the fact that some of the subjects may have started self-medication prior to the submission of specimens. These findings would appear to confirm the opinion expressed by several of the writers cited earlier that many mild diarrheas can be shown to be associated with *Shigella* organisms if they are subjected to laboratory examination and that terms like "Gyppy tummy" should be discarded or at least not considered as describing something other than shigellosis.

The etiology of the episodes in which *Shigella* were not demonstrated is open to speculation. The presence of *Shigella* probably could have been shown in additional cases by replicate cultures. *Salmonella* food poisoning is not unknown in Egypt but these organisms apparently were of no importance in this particular group of cases. The suggestion by Gunn (1946) and by Kean and Smillie (1954) that the diarrhea of new residents and travelers may be due to the ingestion of toxins such as those produced by staphylococci cannot be ruled out by the evidence of this study but it is felt that episodes of "Gyppy tummy" generally do not follow the epidemiological pattern of staphylococcal food poisoning. The presence of *Proteus* and paracolon organisms, while suspected of some slight pathogenicity upon occasion, could not be definitely associated with such sporadic episodes of diarrhea. Only a minority of the cases could have been attributed to intestinal protozoa and none to helminths.

The incidence of non-diarrheal *Shigella* infections among the control group was comparable to that found by Hardy and Watt (1944) in a sample of the general population of the United States.

It was interesting that the percentage of diarrheal episodes associated with *Shigella* in this group of American subjects was approximately the same as that found in Egyptian children (Higgins, Floyd and Kader, 1955). *Shigella* species and serotype distribution was comparable to that noted in Egyptian children (Floyd, 1954).

SUMMARY

The etiology of 226 episodes of mild diarrhea ("Gyppy tummy") among Americans in Cairo, Egypt, was investigated bacteriologically. Sixty-nine (30.5%) were found to be associated with the presence of *Shigella* organisms. *Shigella* were recovered from 79 (25.9%) of 305 cultures from these cases while only 8 (3.9%) of 201 cultures from non-diarrheal patients were positive. Intestinal protozoa were rarely recovered and *Salmonella* and helminths were not found among these cases.

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ON THE INFECTIVITY OF THE FORMOSAN STRAIN OF *SCHISTOSOMA JAPONICUM* IN *HOMO SAPIENS*[1]

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Previous epidemiological studies on schistosomiasis japonica in Taiwan (Formosa) indicated that *Schistosoma japonicum* on that island is very probably a non-human or zoophilic strain (Hsü *et al.*, 1952, 1954, 1955). Experimental studies have also shown that the Formosan strain of this parasite is less infective in the Formosan monkey, *Macacus cyclopis*, than the strains from Japan and the Philippines (Hsü and Hsü, 1956). It was decided that final proof of the inability of the Formosan strain to infect man could be obtained only by exposing human volunteers to this strain. The present paper gives the results obtained from such a study.

MATERIALS AND METHODS

Five male human volunteers were used for this experiment and will be named as Volunteers 1–5 throughout this paper. Their ages varied from 24 to 32. All came from the Chinese Mainland and had been in Formosa for five years. They had no previous history of schistosomiasis and three pre-exposure stool specimens from each man, examined by the sedimentation method, were negative for schistosome eggs. They were also negative for intradermal reaction with *S. japonicum* antigen. Red-cell, white-cell and differential counts were made previous to the day of exposure.

Mature cercariae of *S. japonicum*, obtained from 10 infected snails, *Oncomelania formosana*, collected from the Changhua endemic area were used for exposure. The exposure to the cercariae was made by applying known numbers in drops of water to the volar surface of the arms of the volunteers. Volunteers 1–5 were exposed to 400 cercariae each. Volunteer 5 was first exposed to 400 cercariae but he received an additional 400 cercariae 18 hours after the first exposure. Albino mice were used as control animals in order to assure the infectivity of the cercariae as well as the bisexual composition of the cercaria mixture. All the control animals were found to produce *S. japonicum* eggs and had approximately equal numbers of male and female worms at autopsy.

After the exposure, all the volunteers were confined in a hospital. Daily temperatures were recorded and symptoms and signs were observed. Blood counts were made two to three times a week; stools were examined by the sedimentation method every day; and the intradermal test with *S. japonicum* antigen[2] was

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[2] See Hsü, Hsü and Ritchie, 1955 for the method and interpretation of intradermal reactions.

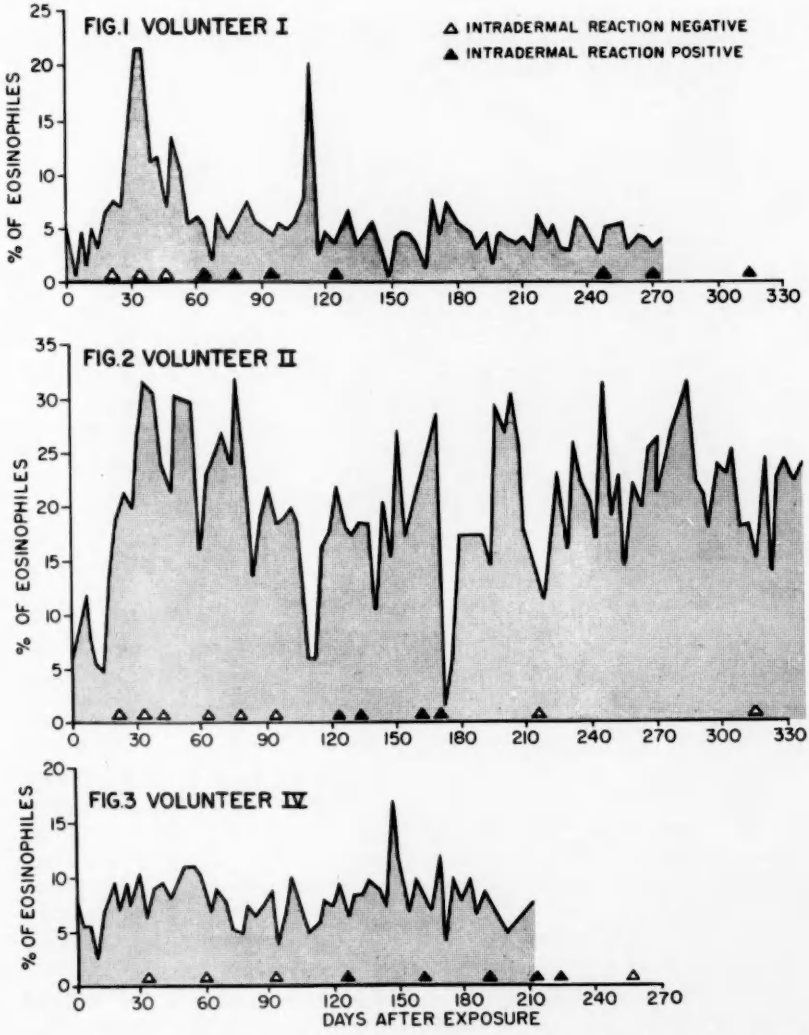
performed once every month. Liver biopsy by laparotomy was done on Volunteers 1 and 5. For Volunteers 1–5 respectively the number of days under study was 314, 336, 204, 256, and 290 days.

RESULTS OF EXPERIMENTS

Considerable variation in the reactions to the exposures was observed in the five men. The volunteers did have the following in common: after exposure and throughout the study urticaria was not observed; liver and spleen were not palpable; no significant changes were seen in temperature, body weight, and total red and white cell counts; and no *S. japonicum* eggs were found in the feces. The other observations on them can best be given individually.

Volunteer 1. At the time when cercariae were applied, there was a transitory itching that subsided in one hour. Erythema was observed at the site of penetration one hour after the exposure and lasted for 10 hours. Papules, measuring one to one and one-half mm. in diameter, appeared on the second day at the site. These persisted for three days and had entirely disappeared by the seventh day. The patient complained of acute abdominal pain on the sixth day. It was not intense, lasted for 15–30 minutes and recurred one to three times a day for a period of three months. The attacks were more frequent during the first two months. The pain was localized in the upper abdominal region and was accompanied by epigastric discomfort. Headache and nuchal pain, accompanied by a dry cough and swelling of the sublingual lymph glands, began on the 27th day and lasted for five days. There were no rales. Before exposure the eosinophils were four per cent. They began to increase in number on the 18th day and reached 21.5 per cent on the 32nd day (Fig. 1). With the exception of a sharp temporary increase on the 112th day, the eosinophil count was within normal limits after the 53rd day. The intradermal reaction was negative through the 45th day but was positive on the 63rd and remained so until the 314th day when the study was terminated (Fig. 1). A liver biopsy by laparotomy was taken on the 146th day. No macroscopic changes were observed. Schistosome eggs were not found in the KOH digestion of two ccm of the tissue. Histologically the sections showed essentially normal architecture. There were slight to moderate periportal fibrosis and slight periportal mononuclear infiltration (Fig. 4). Small foci of parenchymal infiltration which appeared to be independent of the portal area or central vein were observed (Fig. 5) and foci showing areas of coagulative necrosis were also seen. Some vessels appeared to be thickened and dilated. There was a paucity of eosinophils and polynuclear leukocytes. No parasites, eggs or pigment could be identified.

Volunteer 2. Itching was present only as long as the water containing the cercariae remained on the arm. Erythema appeared after it had dried and on the second day small, red papules, one to one and one-half mm in diameter, were evident. The latter disappeared on the 9th day. There were complaints of dull abdominal pain on the fourth day and this continued for about six months. These attacks occurred once or twice a day, especially in the evening and lasted one to two hours. At times, during the second month, the pain was severe. There



FIGS. 1–3. Graphs showing results of eosinophile counts and intradermal reactions of Volunteers 1, 2, and 4.

was a feeling of pressure and fullness in the upper abdomen. Nausea and anorexia was present occasionally during this period. The pre-exposure eosinophil count was six per cent. By the 35th day it had reached 31.5 per cent (Fig. 2). From this point to the end of the observations on the 336th day, the eosinophilia fluctuated between one and 31.5 per cent but the average was well above normal.

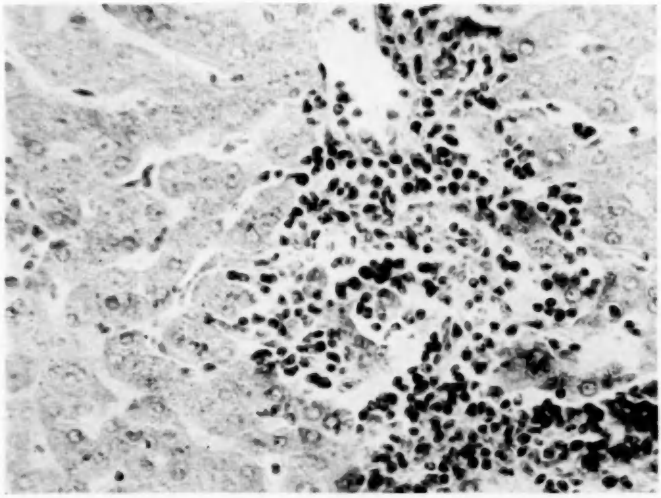


FIG. 4. Section of liver, biopsy material, Volunteer 1, 146 days after exposure, showing periportal mononuclear infiltration.

It was 24 per cent when the last count was made. The intradermal reaction became positive on the 123rd day, remained so through the 170th day but was negative from the 215th day on (Fig. 2).

Volunteer 3. About 15 minutes after the exposure, there was an itching that subsided in two and one-half hours. No erythema was observed but macules appeared in 30 minutes; they became very distinct in one hour. There was an itching again eight hours after the exposure. Papules, two to three and one-half mm in diameter were observed at the site in 17 hours and became more prominent on the second day. They were red and indurated, accompanied by secondary itching. On the fourth day, the papules began to recede and there was no more itching. The patient complained of vague abdominal pain on the 12th day; it was mild, lasted for a short while and recurred once or twice a day for a period of two and one-half months. Occasionally it was accompanied by abdominal distensions. Before exposure, the proportion of eosinophils was 16.5 per cent. After the exposure, throughout the 202 days of study, no significant increase of eosinophil counts could be ascertained except on two occasions when the counts reached 26.5 per cent (on the 29th day) and 28.5 per cent (on the 212th day). The intradermal reaction became positive on the 95th day and remained so through the 125th day. When the last test was done on the 204th day it became negative again.

Volunteer 4. Itching was felt 15 minutes after the exposure and subsided in two and one-half hours. Erythema appeared in one hour and disappeared after two hours. Red macules were evident at the site in two hours. Small papules, one to two mm in diameter, were observed on the second day, and began to re-

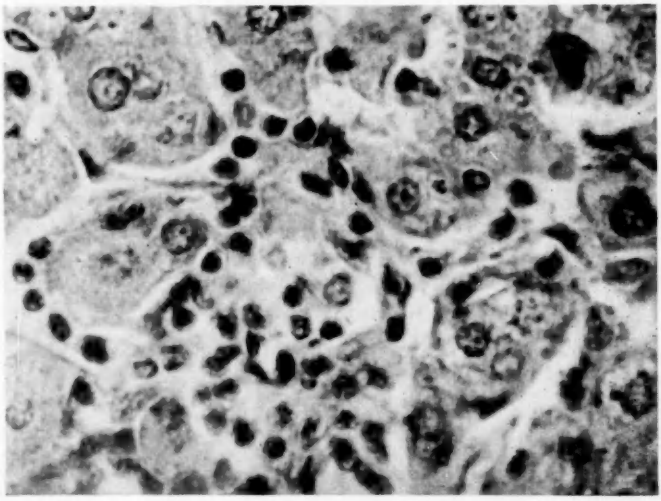


FIG. 5. Section of liver, biopsy material, Volunteer 1, 146 days after exposure, showing parenchymal infiltration.

cede on the third. Beginning from the eighth day, the patient complained of vague abdominal pain for five months. The attack lasted from a half hour to two hours, recurring one to three times a day and became more often and more severe in the second month. It was accompanied by headache, nausea, anorexia, chest pain and general malaise. Before exposure the eosinophils were eight per cent. They remained at about this level throughout the period of study (212 days) except for the count made on the 146th day when it reached 16 per cent (Fig. 3). The intradermal reaction became positive on the 125th day and remained so through the 223rd day. It became negative on the 256th day (Fig. 3).

Volunteer 5. After applying 400 cercariae in drops of water on the right arm, itching was felt after 15 minutes, subsiding in one and one-half hours, and was felt again after six hours. Red macules were observed at the site in 20 minutes. No erythema was seen. A portion of water on the arm dropped off after 25 minutes. Eighteen hours after the first exposure, another 400 cercariae were placed on the left arm by the same method. Fifteen minutes after the second exposure there was again an itching that subsided in 15 hours. Macules were evident in 40 minutes. On the second day, papules, measuring one to two mm in diameter, were observed on both arms, being more prominent at the site of the second exposure. On the third day, papules of the first exposure had almost entirely receded and those of the second exposure began to recede too. On the eighth day no more residue of pigmentation caused by cercaria penetration was seen. Beginning from the 25th day the patient complained of occasional light abdominal pain, accompanied by general malaise and anorexia. In the third month, there was no more abdominal pain but occasional abdominal discomfort

which disappeared in the fourth month. The pre-exposure eosinophilic proportion was six and one-half per cent; by the 26th day it had reached 25.5 per cent. The count was within normal limits after the 83rd day. The intradermal reaction was negative throughout the period of study (290 days). A liver biopsy by laparotomy was taken on the 89th day. No macroscopic changes were observed. Schistosome eggs were not seen in the KOH digestion of 2 ccm of the tissue. Histologically only mild chronic inflammatory reaction was observed in the portal areas with minimal increase in connective tissue. The inflammatory cells consisted of lymphocytes and large mononuclear cells. Pigment was not present in the Kupffer cells. Neither parasites nor eggs could be found.

DISCUSSION AND CONCLUSION

The presence of macules and papules at the site of exposure in all the volunteers indicated that the Formosan strain of *S. japonicum* cercariae had definitely entered the skin. In Volunteers 1, 2, 4 and 5, the reactions encountered during the cercarial penetration were mild and innocuous, corresponding to Type I or II as described by Olivier (1949). In Volunteer 3, the reactions were stronger, tending to approach typical schistosome dermatitis in severity and can be classified as Type III. The lesions produced in these cases were reactions to the exposure of a large number of cercariae (400) which were applied on a very small area of about 4 ccm. The reactions of this nature resulting from ordinary exposure of considerable body surface to a few cercariae would probably go unnoticed or unrecognized. In sensitized persons this might not be true.

Acute abdominal pain seems to be an important symptom encountered in all five volunteers after exposure to this particular strain of parasite. Its first appearance was variable, from the 4th to the 25th day, and lasted for a period of two to five months. The pain was accompanied by occasional nausea, anorexia, epigastric discomfort, abdominal fullness and general malaise. All these symptoms were probably due to the metabolic products of the developing worms. Since there was no evidence of eggs being produced, none of the symptoms seen can be ascribed to them. After the infection of the human strain of *S. japonicum*, all the above mentioned symptoms have been described as common complaints in the early period of the disease in cases among American and Australian troops on Leyte, P. I. in the Second World War (Billings, *et al.*, 1946; Dakin and Connelian, 1947; Faust, *et al.*, 1946; Hunt, 1945, Johnson and Berry, 1945; Thomas and Page, 1945).

Eosinophilia appeared in three of the cases (1, 2 and 5) but it was not a distinct entity in the other two men. In the cases of schistosomiasis japonica in American troops in World War II, Winkenwerder, *et al.* (1946) also noticed the absence of eosinophilia in an appreciable number of their cases. This and the present studies indicate that one can expect considerable individual variations in this and other responses to schistosome infections. The eosinophilia in the above-mentioned three cases began to show up in from one to two weeks after exposure and reached their climax in from 26–35 days. After that, it either receded to normal level in the third month with or without a short second rise in the fourth month, or re-

mained at a level for a long period (in Volunteer 2 at least for 336 days). The results of the present study are in favor of the view proposed by Vogel and Minning (1953) that eosinophilia in schistosomiasis japonica is probably an expression of a phase of hypersensitivity in the early period of the infection. The fact that eosinophilia did occur in rhesus monkeys infected only with male *S. japonicum* cercariae (Vogel and Minning, 1953), together with the fact that eosinophilia also occurred in the present volunteers who harbored only immature worms, indicates that this hypersensitivity might be due to the toxic products originating from the worms.

The positive intradermal reactions shown in Volunteers 1–4 provides an explanation for the contradictory results obtained in previous epidemiological studies in the Changhua endemic area, e.g., an examination of stools from 4,197 persons did not reveal *S. japonicum* but the intradermal test on the same group of people showed 8.4 per cent of positive reactors. This and the results obtained from the volunteers indicate that in infections with this parasite, intradermal reactions can become positive without the presence of *S. japonicum* eggs in the body. On the other hand, the data from Volunteer 5 indicate that an infection where eggs are not produced may not always cause an intradermal reaction.

The histopathological changes in the livers as found in Volunteers 1 and 5, show a certain similarity to the changes produced by unisexual infection of male *S. japonicum* worms in mice as described by Meleney, *et al.* (1952), although such changes can not be regarded as pathognomonic of schistosomiasis japonica.

The negative findings of *S. japonicum* eggs in the stools of all five volunteers throughout daily examinations by the sedimentation method for a period of 204–336 days definitely prove that the Formosan strain of *S. japonicum* can not reach its mature stage in men after exposure. The negative findings of either eggs or pigment of the parasite in the liver give further proof that the cercariae of the Formosan strain did not succeed in developing into mature worms in these volunteers. Other positive findings such as abdominal colic, positive intradermal reactions, eosinophilia, and histopathologic changes in the liver, indicate that this strain developed and remained in these volunteers for some time. It is impossible to determine how long they lived or the stage reached. This and the data reported in previous papers confirm the view that *S. japonicum* in Formosa is a non-human, zoophilic strain.

SUMMARY

Four human volunteers were exposed to 400 cercariae, and one to two batches each of 400 cercariae, of the Formosan strain of *S. japonicum*. Acute abdominal pain, positive intradermal reactions, eosinophilia and histopathologic changes in the liver tissue similar to those produced by unisexual infection of male worms were found in due time after the exposure. Using the sedimentation method, daily stool examinations were negative for *S. japonicum* eggs throughout the study period of 204–336 days and liver biopsies showed negative findings of schistosome eggs and pigment. It is concluded that the Formosan strain of *S. japonicum* develops for a short period in the viscera of man but does not reach maturity. It must be regarded as a non-human, zoophilic strain.

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EXPERIMENTS IN THE THERAPY OF HUMAN TRICHURIASIS AND HOOKWORM DISEASE

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Although the recently demonstrated efficacy of the piperazine derivatives in human ascariasis has vastly simplified the management of this roundworm infection, its companions, trichuriasis and hookworm disease, continue to be therapeutic problems even though the tetrachlorethylene treatment of hookworm infection has given good results in some hands. In an effort to find an effective anthelmintic in these two infections, we have clinically tested twelve drugs, alone and/or in various combinations. Essentially, this is a report of failure to obtain outstanding results with any of these compounds or combinations. It is felt that this negative result will be of value to other investigators and clinicians.

MATERIAL AND METHODS

A previously reported group of trichuriasis patients treated with *leche de higuerón* in Panamá (Hoekenga, 1949) has been included here for comparative purposes. Except for this one group, the studies were conducted in the United Fruit Company (Tela Railroad Company) hospital and schools in La Lima, near the north coast of Honduras, and in several nearby banana labor camps. The various anthelmintic compounds and combinations which were studied are listed in Table 1. Adequate post-treatment follow-up was obtained in 734 *Trichuris trichiuris* and 459 *Necator americanus* infections. This post-treatment follow-up included fecal examinations ten days after completion of treatment and again two to four weeks after treatment. Egg counts were not done except in the leche de higuerón group. The sole criterion of cure was the complete absence of eggs from concentrated (zinc sulfate flotation) specimens on both of the post-treatment specimens. In addition, the post-treatment examinations included the careful examination of direct smears from almost all specimens.

RESULTS

The various anthelmintics and dosage schedules are listed in Table 1, together with the results obtained in terms of cure. It is noted that although some of the cure rates are moderately effective, none of them are truly remarkable on any of the treatment regimens, either in trichuriasis or in hookworm infection. This includes the more or less standard anthelmintics such as hexylresorcinol and a combination of tetrachlorethylene with chenopodium, as well as new hopefuls such as the piperazine salts.

With several of the compounds employed, when cure rates approached 40 to 50 per cent, drug toxicity became significant and precluded an increase to a more effective dosage. The most marked toxicity was the conjunctivitis and keratitis encountered with phthalofyne (Whipcide®); this has been separately

TABLE 1

Experiments in the therapy of human trichuriasis and hookworm disease

Drug	Dose	Age Group	Fasting State?	Post-Treatment Purgation?	Trichuris		Hookworm		Toxicity
					Number treated	Per cent cured	Number treated	Per cent cured	
Piperazine citrate (syrup or capsules)	3.0 gm. in single dose	Grades 2, 5	No	No	66	30.3	34	5.9	Occasional slight dizziness of short duration on all of the schedules
	2.0 gm. daily for 2 days	Grade 6	No	No	29	6.9	15	6.6	
	3.0 gm. daily for 2 days	Grade 6	No	No	13	38.4	8	37.5	
	2.0 gm. daily for 3–6 days	Adults	No	No	33	15.1	52	5.7	
	2.0 gm. daily for 6–10 days	Adults	No	No	15	26.4	22	0.0	
	2.0 gm. daily for 14 days	Grades 1, 2, 3	No	No	8	37.5	8	50.0	
Piperazine adipate (suspension) (tablets)	2.0 gm. daily for 14 days	Grades 1, 2, 3	No	No	9	11.1	9	22.2	None
	3.5 gm. in single dose	Grades 1, 2, 3	No	No	22	50.0	23	9.1	
Piperazine phosphate (suspension)	2.0 gm. daily for 14 days	Grades 1, 2, 3	No	No	9	8.8	7	0.0	None
	3.5 gm. in single dose	Grades 1, 2, 3	No	No	22	36.4	15	6.7	
Piperazine citrate and hexylresorcinol	3.0 gm. piperazine citrate (syrup) and 0.6 gm. hexylresorcinol (capsules) in single mid-afternoon dose	Grade 2	No	No	34	35.3	16	12.5	Occasional slight dizziness and vomiting
Piperazine citrate and methylbenzene (toluene)	3.0 gm. piperazine citrate (syrup) and 2.5 gm. methylbenzene (capsules) in single mid-afternoon dose	Grade 3	No	No	29	24.1	16	0.0	Frequent dizziness, lassitude, weakness, bad taste in mouth
	2.0 gm. piperazine citrate (syrup) and 1.5 gm. methylbenzene (capsules) each afternoon for 2 days	Grade 6	No	No	38	23.7	28	14.3	Occasional slight dizziness
	3.0 gm. piperazine citrate (syrup) and 1.0 gm. methylbenzene (capsules) each afternoon for 2 days	Grade 6	No	No	14	28.5	14	7.1	Occasional slight dizziness
Methylbenzene (toluene)	Total dose of less than 185 mg/K (average 161 mg/K) in divided doses on three successive nights	Adults	No	No	29	27.6	22	50.0	50 per cent with dizziness, gasoline taste, weakness, anorexia and slight headache
	Total dose more than 185 mg/K (average 256 mg/K) in divided doses on 3 successive nights	Adults	No	No	28	21.4	21	33.3	Almost 100 per cent with same symptoms as above
	8.0 gm. (average 133 mg/K) in single early morning dose	Adults	Yes	No	8	37.5	6	33.3	100 per cent with same symptoms as above

	For weight over 60 lb, 2 gm. daily for 2 afternoons; for weight 30–60 lb, 1 gm. daily for 2 afternoons	Adults and children	No	No	6	33.3	7	0.0	20–30 per cent with same symptoms as above
	For weight over 60 lb, 2 gm. daily for 3 afternoons; for weight 30–60 lb, 1 gm. daily for 3 afternoons	Adults and children	No	No	46	26.1	37	5.4	20–30 per cent with same symptoms as above
Vermiplex® (0.5 gm. methylbenzene and 0.5 gm. diphenthane—70 per capsule)	One capsule for each 8 to 10 pounds of body weight	Adults and children	Yes	No	27	18.5	19	31.6	About 40 to 60 per cent with mild dizziness and gasoline taste in mouth
Activated papain (Nematolyt®)	3 bags, taken in fasting state, during 2 day non-protein diet	Adults	Yes	Yes	15	33.3	10	60.0	None
Phthalofyne (Whipcide®)	100–300 mg/K in single dose.	Adults	No	No	35	54.3	—	—	29% with conjunctivitis, usually mild, but severe and with keratitis when dose is more than 200 mg/K. Rare deafness
	100–200 mg/K in single dose	Adults	Yes	Variable	32	40.6	—	—	19% with conjunctivitis and/or keratitis, usually mild, but occasionally severe
	20–145 mg/K daily for 5 days	Adults	No	No	32	25.0	—	—	Infrequent nausea and buzzing in the ear
	200–250 mg/K in 150 cc water as retention enema, at night	Adults	No	No	6	0.0	—	—	None
Sodium santoninate	500 mg. in single dose	Adults	No	Given in cascara syrup	14	14.3	—	—	None
	200 mg. daily for 6 days	Adults	No	Given in cascara syrup	8	37.5	—	—	None
Hexylresorcinol (Crystoids®)	1.0 gm. for adults, lesser amounts for children	Adults and children	Yes	Yes	50	36.0	40	25.0	None. Occasional vomiting of the purge
Tetrachlorethylene and oil of chenopodium	Tetrachlorethylene 2.7 cc and oil of chenopodium 0.3 cc in single dose, combined	Older children and adults	Yes	Yes	30	50.0	30	50.0	None significant. Occasional vomiting of the purge
Leche de Higuerón*	2 oz., diluted in milk for the adult dose; lesser amounts for children	Adults and children	Yes	Yes	27	18.5*	—	—	Minor gastrointestinal irritation in 50 per cent

* Egg counts were done on these patients treated with *leche de higuerón*; the average reduction of eggs in all cases was 62.8 per cent. Only in 5 cases with very low infection was there cure, however.

reported on (Hoekenga 1956). Methylbenzene also gave a high percentage of toxic reactions—chiefly dizziness, weakness and "gasoline taste".

COMMENT

It must be admitted that if egg counts had been done on all specimens, there might have been variations in the statistical results herein presented. First of all, there might have been fewer total cures reported because such examinations might have been conducive to more thorough searching of specimens in some instances. But, on the other hand, with some of the compounds we might have realized significant reduction of worm burdens without actually achieving cure. For instance, as noted in Table 1, the average reduction of eggs in the trichuriasis patients treated with *leche de higuerón* was 62.8 per cent; yet the cure rate was only 18.5 per cent. This might be true of some other vermifuges, and should be investigated in a location where egg counts can be done.

Since none of these treatment regimens was outstanding, the designation of a "drug of choice" has limited meaning. While continuing the search for a more effective compound, we will continue to employ the combination of tetrachlorethylene 2.7 cc. and oil of chenopodium 0.3 cc. as the routine anthelmintic in these infections, with hexylresorcinol as an alternative. In using tetrachlorethylene, we insist on three basic precautions: (1) it should not be given when ascaris are also present, because the ascaris adults may be incited to migrate about, possibly causing an intestinal obstruction; (2) it should not be given simultaneously with alcohol, fats or oils, since these substances increase the absorption of the drug, thereby increasing its toxicity; (3) it should not be given to patients with liver disease. The treatment is given in the fasting state, and in our regimen is usually followed by a saline purge in three hours. Carr and co-workers (1954) have recently shown that tetrachlorethylene is equally effective without post-treatment purgation in hookworm infections, and that, indeed, the adult dose can be increased to 4.0 cc. or 5.0 cc. without encountering significant toxicity. We have employed this treatment regimen in only a few patients.

SUMMARY

Seven hundred and thirty-four trichuris and four hundred and fifty-seven hookworm infections were treated on various anthelmintic regimens involving the use alone and/or in combinations of piperazine citrate, piperazine adipate, piperazine phosphate, hexylresorcinol, methylbenzene, diphenthane-70, activated papain, phthalofyne, sodium santoninate, tetrachlorethylene, oil of chenopodium and *leche de higuerón*. Although some of the results are promising, such as those with the piperazine salts, none are considered really outstanding, cure rates being fifty per cent or less. There is a great need for more effective anthelmintic agents in trichuriasis and hookworm disease; in the meantime, our best results are obtained with a mixture of tetrachlorethylene 2.7 cc. and oil of chenopodium 0.3 cc.

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INSTITUTIONAL PROPHYLAXIS OF ENTEROBIASIS

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Pinworm infection has for years been a major problem in custodial institutions, orphanages, and other similar places. The ease of transmission, difficulty of securing complete cures of patients to eliminate sources of reinfection, toxicity of medications, and many other factors have made the eradication of these parasites in crowded quarters very difficult, and may often have prevented concerted efforts at control.

Many drugs have been used in the treatment of pinworms with varied success. These include among others garlic (Bumbalo and Gustina, 1954), Papain, Egressin, Diphenan, gentian violet, the "Mycins", and Cremothaladine. The results have been good with some drugs, poor with others. In reported tests, Papain (Bumbalo *et al.*, 1953b) was effective in 10 per cent of the cases treated; Egressin (Bumbalo *et al.*, 1953a) in 54 per cent; Diphenan with hexylresorcinal enemas (White and Standen, 1951) in 70 per cent; gentian violet (Standen, 1953) in 85 per cent. All of these drugs have one or other of the disadvantages of ineffectiveness, minor side reactions or expense. Recently the studies of White and Standen (1951), Bumbalo *et al.* (1953, 1954) and Brown and Chan (1955) have demonstrated the marked effectiveness of piperazine citrate against pinworms, and in view of the ease of administration of this syrup and the lack of toxicity in therapeutic doses, it was decided to use it in an attempt to render and maintain a cottage in a mental institution pinworm free.

It was thought that by giving a small amount of drug periodically over a long time, reinfection might be controlled in an institution where proper eradication of all sources of reinfection was almost impossible. This would require a drug of low toxicity, easily taken and tolerated. The piperazines were used in the early 1900's for gout, as early as 1949 for *Ascaris*, and in 1951 for pinworms. All chemical, pharmacological and toxicological reports indicated that it was an extremely safe drug. Recent reports have shown it to be as effective, if not more effective, than any other drug available. A cure rate of 97 per cent was reported by Bumbalo *et al.* (1953b); Brown and Chan (1955) who gave the drug for 14 consecutive days, obtained cures in 100 per cent of their cases.

METHODS

For the purpose of this study, a cottage of 40 typical patients of the institution was selected. Cellulose tape preparations were taken from each patient approximately three hours after going to bed. These were repeated every evening for ten days by the same person, and examined daily except Sunday by the same technician. Of the 40 patients on this ward, 37 gave at least one positive examination and 35 were considered to be heavily infected, showing 10 or more eggs per field.

TABLE 1

Results of treatment of 37 enterobiasis patients with piperazine citrate

	Before Treat.	6 Days After Treat.*	3 Mo. After Treat.†	4 Mo. After Treat.	5 Mo. After Treat.	6 Mo. After Treat.	9 Mo. After Treat.	1 Yr. After Treat.	14 Mo. After Treat.
Number of patients negative	0	24	34	35	36	37	37	37	37
Per cent of patients cured		65%	92%	95%	97%	100%	100%	100%	100%

* 1.0 gm. piperazine citrate twice daily for 6 days.

† 1.0 gm. piperazine citrate twice daily on 2 consecutive days each week for 14 mos.

Treatment was then started with 1.0 gm. of piperazine citrate[1] twice daily for 6 days (equivalent to 1.0 gram piperazine hexahydrate twice daily). All patients weighed over 75 pounds. The drug was given as an orange flavored syrup which was readily taken and well tolerated, with no side reactions. The only difficulty encountered was keeping the child from taking too much medicine.

At the end of the six days' treatment, cellulose tape swabs were taken daily for fourteen days. Any child with one positive tape was considered positive. The children were tested again at the end of three weeks after treatment by taking tapes for seven days. The cure rate on this group was 67.5 per cent at this time (Table 1).

The children were then given 1 gram twice a day for two consecutive days each week. This has been continued for fourteen months. There have been no ill effects from the prolonged treatment. Blood counts, urine tests, monthly weights, etc., have been done and the patients checked physically at least once a month. No special hygienic methods were used to prevent reinfection other than routine cleanliness. The patients occasionally missed doses of the medicine while on vacations or absent from the ward for various reasons.

The first post-treatment check up was made three months after treatment was started, and has been repeated every month since. Tapes are taken each night for six nights and examined the following day. On the first examination three children were still positive, and thirty-seven were negative. The positive tapes held eggs that appeared abnormal in size and configuration. The tests at four months showed two positive patients and the eggs were few and abnormal. The fifth month showed one positive tape with abnormal eggs, and this patient was treated six days with therapeutic doses of piperazine citrate. All subsequent tests have been negative on all patients, and the drug is being continued.

The untreated control group retained an almost constant infection rate of 85 per cent. A third group with 80 per cent infection was given an initial but insufficient treatment with phthalylsulfathiazole for six days with a cure rate of 45 per cent. At the end of six months, this ward had a 75 per cent infection with pinworms.

Six patients were selected in another ward with a 75 per cent pinworm infection.

[1] Piperazine citrate was supplied in the form of Syrup of Antepar by Burroughs Wellcome & Co.

TABLE 2

Results of treatment with one dose of piperazine citrate per week

Patient	Before Treat.	1 Mo. Treat.	2 Mo. Treat.	3 Mo. Treat.	4 Mo. Treat.	8 Mo. Treat.	1 Yr. Treat.
1	Pos.	Pos.	Pos.	Pos.	Neg.	Neg.	Neg.
2	Pos.	Pos.	Pos.	Neg.	Neg.	Neg.	Neg.
3	Pos.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
4	Pos.	Pos.	Neg.	Neg.	Neg.	Neg.	Neg.
5	Pos.	Pos.	Neg.	Neg.	Neg.	Neg.	Neg.
6	Pos.	Pos.	Pos.	Neg.	Neg.	Neg.	Neg.

These patients were given 2 grams of piperazine citrate once a week for one year, and were checked in the same manner as the others. At the end of four months of treatment, all patients were negative for pinworms and remained so for the rest of the year. Treatment has been stopped for three months and two of these patients are again positive for pinworms.

There is evidence that the drug had effect on other species of parasites. Before the test began there were found on the treated ward 8 cases of *Entamoeba histolytica*, 8 cases of hookworm, 6 of *Trichuris*, 5 of *Strongyloides*, 5 of *Entamoeba coli*, 4 of *Giardia* and 3 of *Entamoeba nana*. A ward survey after approximately a year's treatment, consisting of microscopic stool examinations repeated on 3 consecutive days, showed 2 cases of *Entamoeba histolytica*, 9 of *Trichuris*, and one of *Giardia*. No special treatment was given for these parasites. More thorough examination might have shown a somewhat different picture.

DISCUSSION

Our results suggest that a group of 35 heavily infected children can be freed of pinworms safely in a relatively short time with no special effort other than giving piperazine citrate once or twice a week. This is very important to institutions caring for children where elaborate procedures are prohibitive. The problem of reinfection on visits home, or by other contacts, has apparently been overcome by this procedure.

It should be possible to eliminate pinworms from an entire group by this method. Such a situation could be maintained by treating children returning from home or other visits, for several days. They are usually kept in observation isolation now, and treatment could be accomplished during this time. We are considering such a program at the Florida Farm Colony at the present time. The medication is cheap, easy to give, and free of toxicity in therapeutic doses, so this should be relatively simple.

SUMMARY

When 37 patients whose stools were positive for *Enterobius* were treated with 1.0 gm. of piperazine citrate twice daily for six days, and then 1.0 gm. twice a day for two consecutive days each week for 14 months, 91 per cent were negative after 3 months and all after 5 months; all remained negative after 14 months.

When six patients were given 2.0 gm. of piperazine citrate once a week for a year 3 were negative after 2 months of treatment and all after 3 months, and all of these remained negative for the rest of the year.

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TREATMENT OF OXYURIASIS WITH PYRROVINYQUINIUM CHLORIDE[1] (POQUIL)[2, 3]

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In connection with the chemotherapy program for filariasis during World War II, Lare and Brooker of the Eastman Kodak Company prepared a cyanine dye (pyrrovinyquinium chloride)[4] in 1946 which proved to have anthelminthic properties (Perez-Santiago *et al.*, 1953; Hales and Welch, 1953). Weston *et al.* (1953) tested this substance in mice infected with *Syphacia obvelata* and *Aspiculuris tetraptera* and in rats infected with *Syphacia obvelata*, and studied the toxicity in rats, mice and dogs. It was found to be considerably more effective than gentian violet against the mouse pinworm and to be tolerated well in therapeutic doses. The latter workers considered the possible usefulness of the drug in human oxyuriasis. Subsequent preliminary trial[5] yielded encouraging results. It was felt that a controlled study of the efficacy of pyrrovinyquinium chloride in human oxyuriasis was indicated.

MATERIAL AND METHODS

During the last two years, unselected patients on the children's ward of the Jefferson Medical College Hospital, the Outpatient Department of the Curtis Clinic, and patients referred by their physicians, were studied. Scotch tape swabs were taken in the morning in the hospital or during the visit to the Clinic or by the mothers of the children after the technique had been demonstrated to them. Patients found to have oxyuriasis were re-examined and either kept under observation as controls or treated with pyrrovinyquinium chloride. As many daily swabs were examined as could be obtained during and after treatment. Three positive swabs were chosen as the criterion of infection (Jung and Beaver, 1953) and seven negative swabs as the criterion of cure (Sawitz and Karpinos, 1942).

Pyrrovinyquinium chloride (Poquil[6]) is a cyanine dye known chemically as 6-dimethylamino-2-[2-(2,5-dimethyl-1-phenyl-3-pyrryl) vinyl]-1-methylquinolinium chloride dihydrate. It is reddish brown, only slightly soluble in water, and not appreciably absorbed from the gastro-intestinal tract (Hales and Welch, 1953). Its acute oral toxicity for mice was 7.9 mg./kg. and for rats 161 mg./kg.

[1] The generic name has been changed to pyrvinium chloride.

[2] This study was aided by a grant from the Department of Clinical Investigation, Parke, Davis and Company.

[3] Presented at the Annual Meeting of the American Society of Tropical Medicine and Hygiene, Boston, Mass., November 4, 1955.

[4] U. S. Patent No. 2,515,912.

[5] Parke, Davis and Co., Research Department memorandum, May, 1954.

[6] Parke, Davis and Company, trade name for pyrrovinyquinium chloride.

(LD_{50}'s). In chronic toxicity studies over a 4-week period, mice tolerated 18 mg./kg./day and rats 52 mg./kg./day. When given in larger doses to animals, it caused irritation of the small intestine, vomiting, diarrhea, weight loss, and occasionally a decrease in hemoglobin and the number of red cells or even a hypoplasia of the bone marrow (Weston *et al.*, 1953).

This drug was given to our patients by mouth as an aqueous suspension flavored with raspberry syrup. To avoid possible gastro-intestinal irritation, half of the dye granules had been coated (cellulose acetate phthalate type coating) while the other granules remained uncoated. The dose was 1.0 mg. of the dye per kilogram of body weight three times a day for six days. When no intestinal disturbances were observed in 33 patients and clinical results indicated that a large proportion of the coated material was passed unchanged (Parke, Davis and Company memorandum, May 1954), the coated material was omitted and 0.5 mg. of the uncoated dye granules per kilogram of body weight was given three times a day for six days. Another group was treated with 0.7 mg./kg. three times a day for six days. Deliberately, no advice as to special measures of personal hygiene was given. Measures started previously, such as enemas, perianal ointment, sterilization of bed linen and pajamas, disinfection of the bathroom, etc., were discontinued.

Wherever possible, the peripheral blood was examined before and after treatment as to the amount of hemoglobin, number of erythrocytes and leukocytes, and differential counts. The urine was examined routinely before and after treatment.

RESULTS

It was not the purpose of this study to determine the incidence of oxyuriasis in the 1,129 patients examined. Eggs of *Enterobius vermicularis* were found in approximately one in eight persons on one swab examination (one in each five white persons, and one in each 12 colored). Within each race, males and females were found infected at the same ratio, one in eight.

A total of 123 positive patients was included in this study: 52 hospitalized patients, 41 clinic patients, and 30 patients referred by their physicians. Thirteen patients were between 22 and 42 years of age, the others ranged from 1½ to 12 years of age. Pyrrovinyquinium chloride was given to 105 patients, but was withheld from 18.

Of the 18 patients who did not receive the drug, 15 had at least three positive swab examinations, thus complying with the criterion of infection. Three of these 15 became egg-negative as proved by seven or more negative swabs, a spontaneous cure rate of 20 per cent.

Therapeutic effects. The results are summarized in Table 1.

The enteric aqueous suspension of pyrrovinyquinium chloride, one half of which was coated, the other half uncoated, was given to 33 patients three times a day for six days in doses of 1.0 mg./kg. Ten of these had been found positive on three or more swabs. All ten became negative as proved by at least seven negative swabs. The earliest day on which they became and remained egg-free

TABLE 1

Results of treatment of oxyuriasis with pyrrovinyquinium chloride

Group	Egg-positive On At Least 3 Swabs	Egg-free On At Least 7 Swabs	Remaining Positive
Untreated	15	3	12
Total treated t.i.d. for 6 days	45	45	0
1 mg/kg	*10*	*10*	*0*
0.5 mg/kg	*17*	*17*	*0*
0.7 mg/kg	*18*	*18*	*0*

after the beginning of treatment was the third, the latest the eighth day, an average of five. No failure was observed in the other 23 patients who did not fulfill the criteria of infection or period of observation.

The uncoated aqueous suspension of the drug was given to 33 patients in doses of 0.5 mg./kg. three times a day for six days. Of these, 17 had three or more positive swabs and were observed long enough to be examined at least seven times. All 17 became egg-free. The earliest day on which they became negative was the second, the latest the eighth day, an average of 4.6. No failure was observed in 15 of the 16 patients who did not fulfill the criteria of infection or period of observation. One 10-year-old boy remained positive during, and on the second day after completion of, treatment. No further swabs were obtained.

The uncoated drug was given to 39 patients in doses of 0.7 mg./kg. three times a day for six days. Of these, 18 had three or more positive swabs and were observed long enough to obtain at least seven swabs. All 18 became egg-free. The earliest day on which they became and remained egg-free was the first, the latest the seventh day, an average of 3.3 Two of the cured patients belonged to families with five and six infected members, respectively. The other members had not been treated and no advice as to special hygienic measures had been given. No failure was observed in 20 of the 21 patients who did not fulfill the criteria of infection or period of observation. One remained positive, but was not observed beyond one day after conclusion of the treatment. This was a 6-year-old girl who complained of headaches 5 to 10 minutes after taking the drug and had discontinued medication after only four days.

The total number fulfilling the criteria of infection and period of observation who were treated with pyrrovinyquinium chloride in one of the three regimens was 45. All became negative. Of 27 persons examined long and often enough, eggs were found again in 22. The earliest was the 40th day after treatment. When treated a second time, they remained egg-free for 46 days. However, only six patients were in this group.

Side effects. No untoward symptoms were noted by 103 of the 105 patients treated. One 6-year-old girl complained of headache 5 minutes after taking the drug. The other was a 27-year-old woman with Hodgkin's disease who complained of "sore intestines", gas, nausea, weakness, and had urticaria on forearms and chest. However, she had received also actinomycin C, a blood transfusion, and oral penicillin. There was no noticeable change in her lymphadenopathy.

The blood was examined before and after treatment in 65 patients. In no instance was any change of clinical importance observed. This included the patient with Hodgkin's disease and one with hemophilia. The mean hemoglobin amount was 73.9 per cent before treatment and 72.3 per cent after treatment. It remained unchanged in four patients, increased in 25, and decreased in 36. The mean drop was 1.6, a difference of no statistical significance. The mean number of red cells per cu.mm. was 3,880,000 before and 3,810,000 after treatment. The number remained unchanged in six patients, increased in 26, and decreased in 33. The mean drop of 0.07 was not statistically significant. The respective figures for the leukocytes were 8,230 and 7,870; for the polymorphonuclear cells 47.8 and 48.7 per cent; for the eosinophils 4.4 and 3.7 per cent; for the lymphocytes 46.0 and 45.8 per cent; and for the monocytes 1.5 and 1.4 per cent. In none of these results was the change of statistical significance.

Routine examinations of the urine before and after treatment were made in 58 patients but no clinically important changes were noted.

DISCUSSION

Oxyuriasis presents diagnostic and therapeutic problems which are different from those of other parasitic infections. Neither the presence nor the absence of the infection can be established with certainty. *Enterobius vermicularis* does not commonly lay eggs while in the intestinal tract, but oviposits on the perianal area and dies. The finding of adults or eggs, therefore, signifies a past infection and it may be the exit of the last worm. Jung and Beaver (1953) have shown that eggs must be found on at least three examinations before it can be assumed that infection is present and likely to continue. Sawitz and Karpinos (1942) found that seven negative examinations are needed to assume that the infection is absent. Unless these factors are taken into account, studies on the efficacy of therapy may be misleading. In the present study, 45 patients fulfilled these requirements.

The therapeutic principles of helminthic infections differ from those of other infections, since helminths as a rule do not complete their life cycles and do not multiply in man. In consequence, therapy of helminthiases is satisfactory if some of the worms are removed. Repeated treatment may remove them all. In oxyuriasis, however, reinfection takes place so readily that it behaves as if the worms multiply within the host. Completely effective treatment is required since even rigorous sanitary measures have proved futile in the prevention of reinfection (Sawitz *et al.*, 1940). In a children's home in New Orleans, the incidence of 38 per cent in 69 children rose to 52 per cent after a six-week experiment with sanitary measures.

The required 100 per cent efficacy seems to be fulfilled by pyrrovinyquinium chloride (Poquil). In the present study, no failure was observed in 45 controlled cases and the results obtained in an additional 60 cases are in support of the efficacy of this drug. In addition, the results were obtained without using any deliberate hygienic measures.

While the hospitalized children may have been subjected to measures of

cleanliness, the clinic patients probably did not practice them. Moreover, in two families with five and six infected members, only one member was treated; in both cases cures were obtained. Previously, cleanliness by itself was found ineffectual. The present results seem to indicate that it is not even required when an efficacious drug is given.

Patients observed long enough remained egg-free for a minimum of 39 days. Since the life span of *Enterobius vermicularis* is probably less than this, it can be assumed that the six-day treatment kills them in all the stages present at that time. No information is available on the action of the drug on the eggs. Eggs were found in four instances on the first or second day after completion of medication but not on subsequent examinations. Similar observations on the persistence of eggs for a few days and their subsequent disappearance were made by Jung and Beaver (1953).

When the drug, one half coated, one half uncoated, was given in doses of 1 mg./kg., the patients were found egg-free on the average on the fifth dav. When the uncoated drug was given in doses of 0.5 mg./kg., the average was 4.6 days, and in doses of 0.7 mg./kg. it was 3.3. These results suggest that only the uncoated half was effective. The dose of 0.7 mg./kg. seemed to have a greater potency. However, the differences were found not to be of statistical significance.

Pyrrovinyquinium chloride was 100 per cent effective in our study. On the basis of statistical probabilities, there is a 95 per cent assurance that the treatment is at least 94 per cent effective. In other words, only 5 times in 100 would a treatment with as poor an efficacy as 94 per cent be expected to give 100 per cent cures such as were obtained in our 45 patients.

The structural formula of the drug and the toxicity studies in animals pointed to a potentially toxic action on the hematopoietic system. No difference of clinical importance was observed in the blood before and after treatment in our cases. Since the drug is not absorbed from the gastro-intestinal tract in appreciable amounts (Hales and Welch, 1953), and did not result in changes of the blood count when given in doses more than ten times that used in the present study (Perez-Santiago *et al.*, 1953), it is not likely that depression of hematopoiesis will occur. However, it remains a possibility.

The low solubility of the drug necessitated that it be dispensed as a suspension. The raspberry flavored syrup was taken readily by the adults and all but one of the children.

This study indicates that pyrrovinyquinium chloride (Poquil) in doses of 0.5 to 0.7 mg. per kilogram of body weight three times a day for six days, is a satisfactory treatment for oxyuriasis.

SUMMARY

Of 123 persons with oxyuriasis 60 complied with the criterion of infection, i.e., three positive Scotch tape swabs were obtained. A total of 45 patients were treated with pyrrovinyquinium chloride (Poquil), 15 were not. A minimum of seven negative swabs was chosen as the criterion of cure. Three of the 15 controls became and remained negative on at least seven examinations, a spontaneous

cure rate of 20 per cent. All 45 of the treated group became negative, a cure rate of 100 per cent. These results were obtained without advising the patients to use any special sanitary measures. In two instances, only one member of a multiple-infected family was treated and even then cure resulted. Reinfection became patent at the earliest on the 40th day after completion of medication. No untoward effects of clinical importance were encountered.

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SOME FACTORS INFLUENCING EGG LAYING IN *ONCOMELANIA NOSOPHORA* AND *ONCOMELANIA QUADRASI*, INTERMEDIATE HOSTS OF *SCHISTOSOMA JAPONICUM*[1]

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The control of schistosomiasis has been hampered by the lack of knowledge of the biology of the snail vectors. Most of the previously published information on the egg-laying habits of *Oncomelania* has been obtained from field observations. Because of the difficulties involved, the data were based largely on the finding of broods of young snails, rather than on the eggs.

The eggs of *Oncomelania* snails were first observed by Sugiura (1933). Although there is some doubt about the identity of the initial eggs which he obtained from nature, those he obtained under laboratory conditions were certainly *O. nosophora*. Li (1935) properly identified eggs of *O. hupensis* after previously being in error (Li, 1934). Eggs of *O. quadrasi* were first studied by Sumpaico in 1941 (reported 1947), and later by Abbott (1946). No reports are known to us which indicate that the eggs of *O. formosana* have been found under natural conditions. Additional information on *O. nosophora* was given by Sugiura (1933); Ann. Rept., 406 MGL, 1950–1954; and McMullen (1951), who made life-history and ecology studies. De Witt (1952) reported on the laboratory rearing of the four species of *Oncomelania;* Otori, *et al.* (1953) reported on egg laying and hatching, and Otori and Ritchie (1954), on the biology of the snails.

The eggs are laid singly and covered with a layer of mud-like material which was stated by Abbott to be snail feces. This has been confirmed by Ritchie, *et al.* (1951), but whether the snail uses only feces in the presence of soil is not certain.

The purpose of this paper is to report on the following four experiments: (1) selection of materials for oviposition; (2) effect of water level on rate of reproduction; (3) effects of temperature on reproduction; and (4) relation of oviposition to the water line. Information was obtained for both *O. nosophora* and *O. quadrasi* on all these points.

MATERIALS AND METHODS

The *O. nosophora* used in these experiments were received from Japan, and the *O. quadrasi* came from the Philippines. Only adult snails, after they had become adapted to the laboratory, were used. These were sexed (Wong and Wagner, 1954) and marked with paint. The observations on egg laying and production of young were made over a period of one year. The experimental snails that died during this period were removed and replaced by live ones from stock. During

[1] These investigations were carried out under the sponsorship of the Commission on Parasitic Diseases, Armed Forces Epidemiological Board, Department of the Army, under Contract No. DA-49-007-MD-307.

the course of the experiments about 40 per cent of the snails had to be replaced and the mortality rate by sex was approximately equal.

The containers used for the various experimental groups of snails consisted of unglazed, porous, clay flower-pot saucers measuring either 5 inches in diameter by 1¼ inches deep, or 6 inches by 1¾ inches. A standard mixture of autoclaved soil, sand and gravel, in the ratio of 2:1:1, was placed in most of the saucers. Sufficient water was added to this mixture to produce a firm mud and it was patted into place so that it sloped about one-third of the way up the side of the container. In some saucers only sand and filter paper were used. The water level in the saucers was maintained by placing them in shallow containers filled with water. Filter paper was added as food and replaced periodically. The saucers were covered with glass lids that had been cut to leave a one-inch air space at either side. These spaces were covered with metal screen.

If unfavorable conditions developed in the saucers due to excessive growth of mold, algae and bacteria, replacements were made. Out of 141 saucers used, it was necessary to replace 32 during the course of the experiments. Twenty-six of these were among the saucers kept at the highest temperature.

The examinations for eggs and young snails were made at intervals of about two months. The eggs were not disturbed and the young snails were removed before they reached one-half adult size. The length of the interval between examinations accounts for the smaller numbers of eggs observed, as compared with the numbers of young snails. It allowed many of the eggs to hatch between observations.

For the first two experiments concerned with temperature and deposition of eggs, sets of 15 containers were placed in each of three constant-temperature rooms held at 20°, 26°, and 32°C. Each group of 15 consisted of five sets of three saucers. One set contained only washed sand and filter paper, another contained the standard soil-sand-gravel mixture; and the others contained the standard mixture plus brick, maple leaf, or wood. Each set of three consisted of one 6-inch and two 5-inch saucers. One pair of snails was added to each of the latter, and three males and five females were placed in the former. All containers were exposed to continuous light from a single 24-inch, 20-watt, daylight fluorescent tube at a distance of 9 to 10 inches. The methods used were the same for both species except that the 6-inch saucer containing only the standard mixture was omitted for *O. quadrasi*. Six months after this experiment was started one of the 5-inch saucers was removed from each set for *O. nosophora*, and one of the 6-inch saucers for *O. quadrasi*.

In the third experiment the same temperatures mentioned above were used, but the water levels were varied, and the snails were offered a variety of materials from which to choose egg-laying sites. Soil, gravel, sand, brick, wood, maple leaves, and filter paper were placed in 27 saucers. One-third of these were maintained with a high water level which covered the contents. In another third the level was adjusted so that about one-third of the contents was covered. The remainder of the saucers were kept moist only. Three saucers with each water level were kept in the three different constant temperature rooms. The ratio of

two small saucers to one large one, with the numbers of snails indicated above, was also repeated for each water level at each temperature. Data obtained for experiment four were derived from experiments two and three. The position of the eggs in relation to the water line was noted during the course of these tests.

TABLE 1

Selection of materials for oviposition under diverse temperatures and water levels. Experiment 1, standard materials (filter paper, soil, gravel) in all. Brick, leaf and wood occurred in 3 saucers at each temperature. Experiment 2, all materials appeared an equal number of times

	O. nasophora—Number of Eggs						
	Filter paper	Saucer wall	Soil	Gravel	Brick	Leaf	Wood
Experiment 1							
20°C		9	87	7			
26°C			223	12	2	1	
32°C		1	139	12			
Totals		10	449	31	2	1	
Per cent		2	91	6.3	0.5	2	
Experiment 2							
High*		4	91	3	4	12	
Medium†			123	14	10	1	
Low‡			80	6			
Totals		4	294	23	14	13	
Per cent		1.2	84.5	6.6	4	3.7	
Grand total		14	743	54	16	14	
	O. quadrasi—Number of Eggs						
Experiment 1							
20°C		64	19	21	29	10	
26°C	5	18	13	48	8	2	31
32°C		13	7	11	84	13	
Totals	5	95	39	80	121	25	31
Per cent	1	24	10	20	31	6	8
Experiment 2							
High*		7	14	1	85	10	1
Medium†		4	22	22	24	22	2
Low‡		2	54	35	30	33	9
Totals		13	90	58	139	65	12
Per cent		4	24	15	37	17	3
Grand total	5	108	129	138	260	90	43

* Dish contents flooded.

† Dish filled to ⅓ capacity with water.

‡ Dish contents kept moist.

RESULTS AND DISCUSSION

Experiment 1

Selection of materials for oviposition. In the summary of the results presented in Table 1, it will be noted that approximately 91 per cent of the eggs of *O. nosophora* were found on the soil. Only 6 per cent were found on gravel; 2 per cent on the saucer walls; less than 1 per cent on brick, and leaves; and none on wood and filter paper. Only one young snail was found in the examinations of nine saucers containing only sand and filter paper, and maintained at the three temperatures. This snail was found in a saucer kept at 26°. It will be remembered that in this experiment the snails did not have all the materials present from which to make a choice.

The pattern of egg-site selection by *O. quadrasi* differs from the other species (Table 1). Out of 397 eggs observed, 31 per cent were found on brick, as this species of snail was less specific in its choice of egg-laying sites. The laying of almost one-third of the eggs on the type of substrate offered by brick is surprising when it is considered that only three of the 14 saucers at each temperature contained this material. It will be noted that this species is similar to *O. nosophora* in its reaction to sand. Only seven young developed in the saucers containing sand. All of these were in the 32° room.

It will be noted as regards selection of materials for oviposition in the test on water level (Table 1) that more eggs of *O. quadrasi* were again found on brick than on any other material. As in the previous experiment, this species showed less specific selectivity than *O. nosophora*, but there were certain differences in the pattern. For example, in the first experiment, 24 per cent of the eggs were found on the saucer wall, but in this test only 4 per cent were found in this location.

It appears that when favorable egg-laying sites are not present, egg laying in these snails is almost completely inhibited as shown in the cases where the only material present was sand. This may be because sand is an unsuitable egg-laying site, or it may be due to the absence of food, the absence of material for covering the eggs, or the failure of the eggs to hatch after they were laid.

A correlation appears to exist between these laboratory findings and observations reported on findings of eggs in the field. Since *O. nosophora* lays most of the eggs on soil, it may help to explain why the eggs of this snail have probably not been found in the field, as it is difficult to locate the eggs on soil. As regards the eggs of *O. quadrasi* these have been found in the field on materials such as rotting, water-logged coconut husks, dead leaves, sticks and boards. This agrees with the present findings of these eggs on various materials, especially brick.

Experiment 2

Effect of water level on rate of reproduction. Nearly half of the eggs and young of *O. nosophora* in this experiment were found in containers kept at a medium

TABLE 2

The effect of water levels on the number of offspring produced at different temperatures

Temps., °C.	Water-Levels in Saucers			
	High*	Medium†	Low‡	Totals
	O. nosophora			
20	226	323	39	588
26	251	267	119	637
32	4	0	1	5
Totals	481 (39%)	590 (48%)	159 (13%)	
	O. quadrasi			
20	121	141	132	394
26	139	219	123	481
32	6	59	5	70
Totals	266 (29%)	419 (44%)	260 (27%)	

* Dish contents flooded.
† Dish filled to ⅓ capacity with water.
‡ Dish contents kept moist.

water level. Almost as many, 39 per cent, were found in the saucers with a high water level. Only 13 per cent were found in the saucers where the contents were just moist (Table 2).

The distribution of young snails of *O. quadrasi* in the containers at the three water levels was more nearly equal than in the other species, with 29 per cent found in the saucers with a high water line, 44 per cent in the medium, and 27 per cent in those with a low water level (Table 2).

Both species require water for egg laying and hatching. In the laboratory the optimal amount required is that which will provide standing water in the saucer, but not entirely cover the materials which are used as egg-laying sites. Egg laying and hatching occur at both water level extremes, but in greatly reduced numbers, especially at the low level. This may indicate that insufficient amounts of water may be harmful to egg laying and hatching. The effects of the degree and duration of dryness on egg hatching has not been determined. Complete drying of eggs for one and two days destroyed them. If this usually occurs, drying may prove to be an effective means of destroying *Oncomelania* eggs in the field, provided seasonal conditions and agricultural practices make this possible.

Experiment 3

Effect of temperature on reproduction. The data for this observation are taken from Experiments 1 and 2 as they concern the effect of temperature on reproduction. During the period of one year eggs or young of *O. nosophora* in Experiment 1 were found in 25 out of 45 saucers used. Ten of the negative containers were found among those kept at 20°C. Three of the positive saucers at the

highest temperature contained eggs, but no young developed. Young snails were first observed in two saucers kept at 26°C. about one month after the experiment was started. It was noted that there was a tendency for young snails to appear first at 26°C. During the first two observation periods young *O. nosophora* snails were found in 13 containers, of which nine were in those kept at the medium temperature. In *O. quadrasi* during the same period, 7 of the 14 saucers in which young first appeared were those kept at 26°C.

In the experiment concerned with the effect of water level on reproduction, it was noted that the first young *O. nosophora* to appear in this series of saucers was in one kept at 26°C., one month after the experiment was started. After two months, six saucers at this temperature had young snails, but only one of the nine at each of the other temperatures had them. One saucer at 32°, after two months contained 18 eggs, but these did not hatch. In three saucers kept at 20° young snails were not found until the 12th month. All containers kept at 26° produced young snails, but one at 20° and five at 32° did not. The first appearance of young *O. quadrasi* was similar in pattern to that observed in Experiment 1. Only one container did not produce young, compared to 11 in the first experiment.

It was also observed that more young *O. nosophora* were produced at 26°C. than at the other temperatures (Table 3). Since the pattern of reproduction for

TABLE 3

The number of young snails produced at different temperatures

	Water level	20°	26°	32°
		O. nosophora		
Exper. 1	Medium only	291	1013	254
Exper. 2	High*	226	251	4
	Med.†	323	267	0
	Low‡	39	119	1
Totals		588	637	5
Grand totals		879	1650	259
		O. quadrasi		
Exper. 1	Medium only	152	473	491
Exper. 2	High*	121	139	6
	Medium†	141	219	59
	Low‡	132	123	5
Totals		394	481	70
Grand totals		546	954	561

* Dish contents flooded.

† Dish filled to ⅓ capacity with water.

‡ Dish contents kept moist.

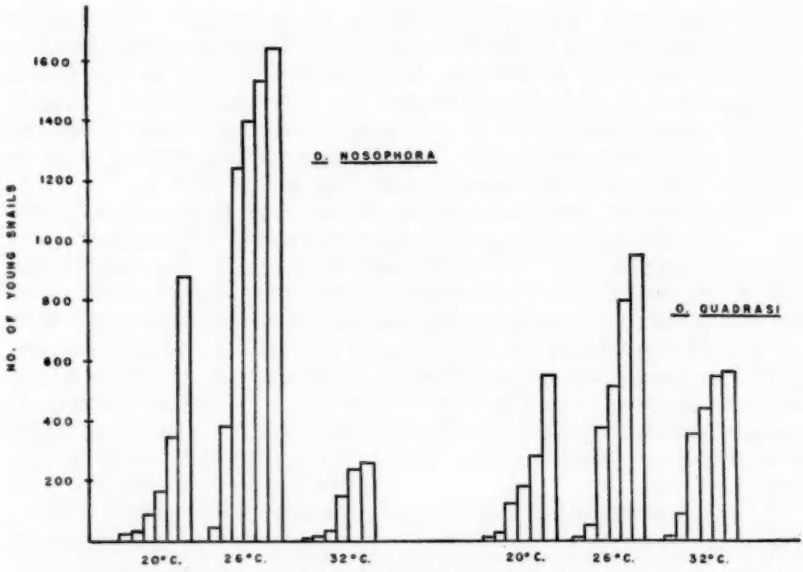

FIG. 1. Total number of young *O. nosophora* and *O. quadrasi* at 3 temperatures. (Cumulative for exp. 1 & 2. Columns indicate total snails found at bimonthly intervals.)

this species was the same in both experiments, the data have been combined in Figure 1. After four months, 1,242 young snails were found in saucers kept at 26°, compared to 85 at 20°, and 26 at 32°. After one year, the counts were 1,650, 879, and 259, respectively.

Data taken from Experiment 1 show that temperature affected reproduction in *O. quadrasi* in a manner similar to *O. nosophora*. However, the effect of the highest and lowest temperatures resulted in about equal numbers of young produced (Table 3). When the results of Experiments 1 and 2 were combined, it was found that by the end of the year almost twice as many snails had developed in the saucers kept at 26° as at either of the other temperatures. As shown in Fig. 1, the pattern of reproduction was somewhat different from that of *O. nosophora*. Early in the experiment in the saucers kept at 32°, the number of snails found approached that found at 26°, and was well above that found at 20°. At the end of the experiments, however, the total numbers of young from the saucers kept at the lowest and highest temperatures were about the same.

As far as the number of young produced in Experiment 2 was concerned, a total of 588 *O. nosophora* was found in the saucers kept at 20°, 637 at 26°, and only five at 32° (Table 2). In this case, the number of snails produced at 20° approached that at 26°, and fewer snails were produced at the highest temperature than in the first experiment.

O. quadrasi gave a pattern similar to the other species in this water level

test; with 394 at 20°, 481 at 26°, and 70 at 32° (Table 2). The data are at variance with those obtained for this species in the combined Experiments 1 and 2 where a slightly greater number was found at 32° than at 20°C.

Generally speaking, snails kept at 26°C. reproduce earlier and greater numbers of young are produced in both species than at 20°C. or 32°C., so this temperature is effective for rearing purposes. For maintaining the snails, 20°C. appears to be the most suitable since it has been observed that the mortality rate is lower than at the other temperatures. Although the reproduction rate was low at the onset, this rate increased rapidly during the last observation period. It was also noted that the saucers did not become fouled with algae and mold and by rapid organic decomposition as occurred especially at 32°C. The highest temperature tested proved to be unsatisfactory for the culture of snails.

Experiment 4

Relation of oviposition to water line. Data for this observation were taken from Experiment 1 and from the saucers with the medium water level of Experiment 2. About 94 per cent of the *O. nosophora* eggs were laid above the water line, 5 per cent at, and 1 per cent below the line. The percentages of *O. quadrasi* eggs found in those positions were 71, 15, and 14 respectively. In Experiment 2, one-third of the saucers had enough water to cover the contents. It will be noted in Table 2 that the number of eggs observed in these containers was rather high. Naturally all of these were laid under water. The eggs that were laid above the water level in the experiment were kept moist by daily spraying and by contact with the water in the saucers.

Since the majority of the eggs of both species are laid above the water level, drying of the soil might serve as the means of control, especially for *O. nosophora* in Japan.

SUMMARY

The egg-laying habits of *Oncomelania nosophora* and *O. quadrasi* were observed experimentally at 3 temperatures over a 12-month period. It was noted that:

1. Most (84.5%–91%) of the eggs of *O. nosophora* were laid in soil, while those of *O. quadrasi* were laid largely on objects such as brick, gravel and saucer wall.

2. A medium water level (⅓ saucer capacity) in the rearing saucers resulted in better snail reproduction than at the high (flooded) or low (moist) level.

3. At 26°C. egg laying started earliest and more eggs and young were found than at 20° or 32°C. Of these last two temperatures, the better results were obtained at 20°C.

4. Snail mortality was lowest at 20° and highest at 32°C. Adverse culturing conditions developed more rapidly in the saucers kept at 32°C. than at the other two temperatures.

5. The majority of the eggs were laid above the water line. For *O. nosophora* the percentage is 94, and for *O. quadrasi* the percentage is 71.

ACKNOWLEDGMENT

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EFFECTS OF WATER LEVEL FLUCTUATION ON EGG LAYING IN *ONCOMELANIA NOSOPHORA* AND *ONCOMELANIA QUADRASI*[1]

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These laboratory studies were conducted to elucidate certain field observations made by workers on egg-laying habits of *Oncomelania*. Abbott (1946), while working with *Oncomelania quadrasi* in the Philippines, observed increased mating activities by this snail during rainy spells, and postulated that the egg-incubation time is influenced by rainfall and flooding. Pesigan and Hairston, of the Bilharzia Control Project, Philippines (1955), have indicated that rainfall stimulates egg laying; and, that for successful breeding to occur, frequent rains over a period of 3 to 4 weeks are necessary. Several investigators have noted that copulation is most common in the spring in areas where the climate induces hibernation in *Oncomelania* (Sugiura, 1933; Mao, 1948; McMullen *et al.*, 1951; Li, 1953; Ritchie, 1955). This is probably due to spring rains, temperature, or the use of irrigation ditches during the spring months. Under laboratory conditions we have observed that Oncomelanid snails will copulate at any time during the year and especially when they are transferred from different rearing dishes and placed together in a Petri dish with water.

The present laboratory study was designed to determine the effect of temporary flooding of the culture-dish contents on egg laying in *O. nosophora* and *O. quadrasi*. The position of the eggs laid in relation to the normal water level and the number of young snails recovered were noted.

MATERIALS AND METHODS

The *Oncomelania nosophora* and *O. quadrasi* used in the experiments came from field collection in Japan and the Philippines, respectively. They had been in the laboratory for several months and were well adapted to the environment. The flooding experiments were conducted over a 12-month period. The tests were repeated three times for each species, the starting dates being the only apparent difference. The colonies were maintained in a constant temperature room at 26° C. and under continuous light (single-line 20-watt fluorescent daylight tubes).

For each test four Pyrex dishes, 9 inches in diameter and 3.5 inches deep, were used as the culture chambers. Each container was supplied with soil-sand mixture, a piece of brick, a piece of wood, maple leaf, and filter paper. Each container was covered with a glass lid. The materials were arranged so that approximately one-half of the surface of each was submerged when the dish was filled to about a quarter of its capacity. Two dishes in each set were used for each species.

[1] This work was conducted under the sponsorship of the Commission on Parasitic Diseases, Armed Forces Epidemiological Board, and was supported by the Office of the Surgeon General, Department of the Army under Contract No. DA49-007-MD-307.

Ten male and 20 female snails were separated (Wong and Wagner, 1954) and placed in each dish. One pair of dishes was used in the flooding experiment and the other pair served as controls. In the latter the water was maintained at the same level throughout the experiment, the dishes being one-quarter full. In the former the dishes were flooded for 18 hours at intervals of approximately two weeks. Weekly examinations were made for eggs with a Zeiss-Opton stereomicroscope. When young snails were found they were removed from the dishes.

RESULTS

The data obtained in the experiments with *O. nosophora* were not always consistent. In the first test the flooded snails produced fewer eggs than the controls (Fig. 1). In the second experiment the reverse was true during the first laying period, but reverted to the former pattern during the second period (Fig. 3). In the third the difference was not significant (Fig. 5). When the data from all three experiments were combined, the total number of eggs produced by the controls was about twice that obtained in the experimental dishes (Table 1). *O. quadrasi* consistently produced more eggs in the experimental dishes than in the controls (Figs. 2, 4, and 6). When the data were combined the ratio was found to be 2.2:1 (Table 1).

Of the 4897 eggs of *O. nosophora* observed, in both experimental and control dishes, approximately 92 percent were laid above the water line. In both groups of *O. quadrasi*, 6940 eggs were seen, and 84 per cent were above the water line.

In all the dishes fewer young snails were recovered than the number of eggs observed. While some shells of young snails that had died shortly after hatching may have been missed, this difference probably indicates the approximate mortality rate during the egg stages, plus the number of infertile eggs. While the number of eggs laid by *O. nosophora* in the control cultures was larger, only 50

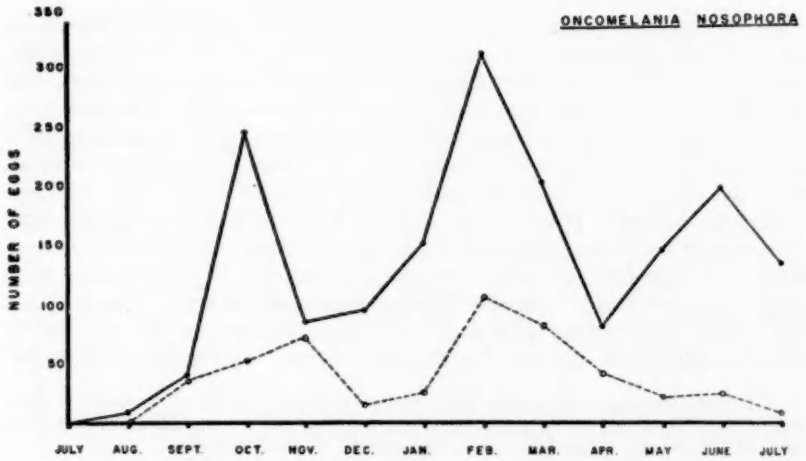

FIG. 1. Number of eggs, control snails - - - - ; test snails ————

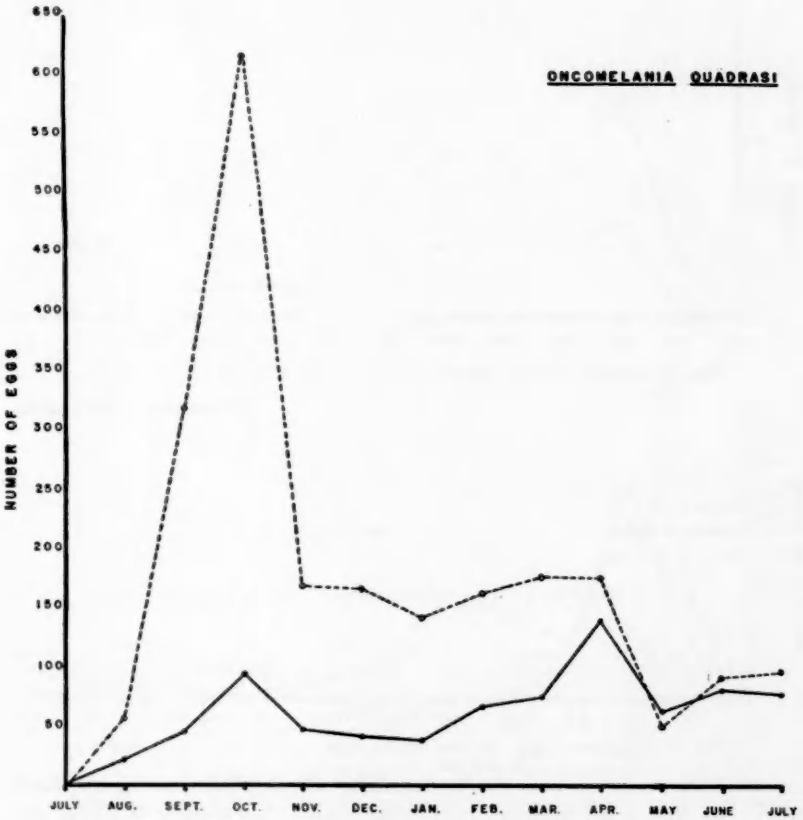

FIG. 2. Number of eggs, control snails - - - - ; test snails ————

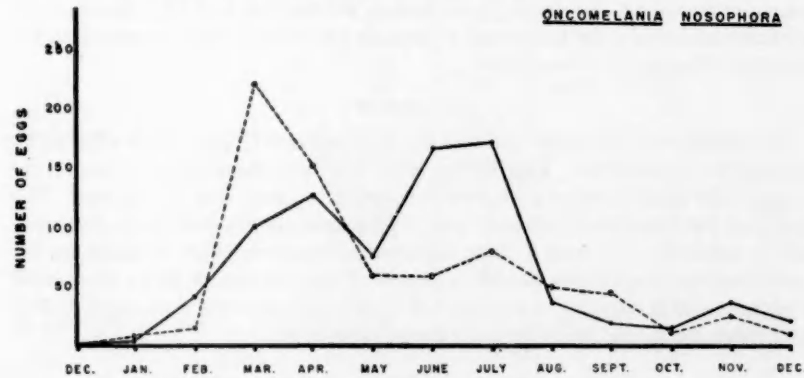

FIG. 3. Number of eggs, control snails - - - - ; test snails ————

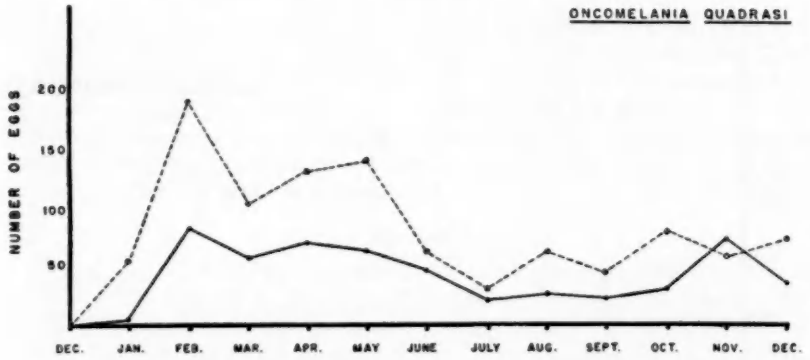

FIG. 4. Number of eggs, control snails - - - - ; test snails ————

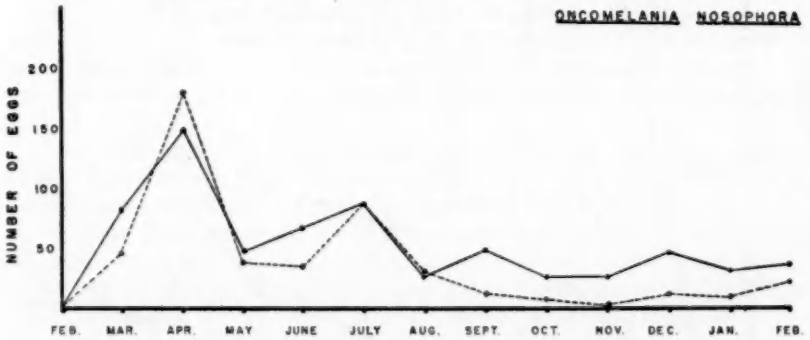

FIG. 5. Number of eggs, control snails - - - - ; test snails ————

per cent of them hatched. In the flooded dishes the number of young recovered was 79 per cent of the total number of eggs, making the number of young produced about the same for the two groups. With *O. quadrasi* the percentage of recovery was about the same, 72 per cent in the controls and 76 per cent in the experimental dishes. In this case 2.4 times as many snails were produced in the flooded dishes as in the controls.

DISCUSSION

It is apparent from these data that in the laboratory *O. quadrasi* is affected by water-level fluctuations. This agrees with the field observations reported by others that rainfall induces increased reproductive activities in this snail. The group at the Bilharzia Control Project, Philippines (1955), points out that since newly hatched snails have a 10 to 12-day aquatic phase prior to taking on the characteristic amphibious nature, a period of approximately 20 to 30 days of frequent rains is required to activate egg laying, to hasten the incubation period, and carry the young snails through the aquatic phase.

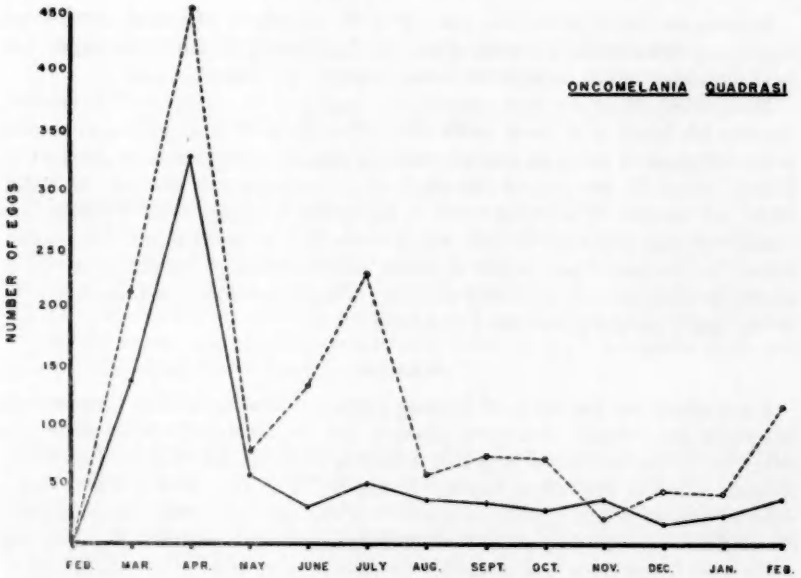

FIG. 6. Number of eggs, control snails - - - - ; test snails ————

TABLE 1

Number of eggs and young snails found in three tests of the flooding experiments

Test	Control Snails			Test Snails		
	Eggs		Young snails	Eggs		Young snails
	Above	Below		Above	Below	
Oncomelania nosophora						
1	1549	152	639	459	27	479
2	740	62	633	676	72	734
3	644	31	309	468	17	140
Total	2933	245	1581	1603	116	1353
Per cent	92.3	7.7		93.3	6.7	
Oncomelania quadrasi						
1	530	259	694	1869	339	2014
2	436	104	595	935	103	1154
3	758	56	256	1453	88	461
Total	1724	419	1545	4257	530	3629
Per cent	87.5	12.5		90.1	9.9	
Grand total	4657	664	3126	5860	646	4982

Reports on the reproductive activity of *O. nosophora* infer that water is an important factor in the life cycle of this snail, although, perhaps, not as great as in *O. quadrasi*, which, unlike the former, occurs in a tropical area.

Mao (1948), reporting on *O. hupensis* in China, and Li (1953), on *O. formosana*, describe life histories of these snails resembling those of *O. nosophora* in respect to the influence of water on reproduction. Li also observed that from January to March, about 75 per cent of the adult *O. formosana* snails were in the water while only about 25 per cent were in the water from April to October. The number of eggs occurring in and out of water in the field has not been determined. In the laboratory where seasonal conditions were essentially avoided, insofar as temperature, light, and amounts of water are concerned, the majority of the eggs were laid above the water level.

SUMMARY

Experiments on the effect of flooding culture dishes containing *Oncomelania nosophora* gave results that were variable, but the combined results showed a reduction in the number of eggs laid. Flooding experiments conducted on *Oncomelania quadrasi* showed an increase in egg laying in the ratio of 2.2:1. *Oncomelania nosophora* laid approximately 92% of the eggs above the water line, and *Oncomelania quadrasi* laid about 84% of their eggs in that position. Fewer young snails were found than eggs, probably because some of these died and the shells were not recovered, and probably because some eggs failed to hatch.

ACKNOWLEDGMENT

The authors gratefully acknowledge the assistance of Dr. Donald B. McMullen who gave extensive assistance in correcting the manuscript. Appreciation is expressed to Dr. L. S. Ritchie and Dr. T. P. Pesigan, who supplied the field collections of the snails from Japan and the Philippines, respectively.

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THE INCUBATION PERIOD OF THE EGGS OF *ONCOMELANIA NOSOPHORA*

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The incubation period for eggs of *Oncomelania* snails has been varyingly reported, without supporting data, to be from 10–15 days. From observations on eggs of *O. nosophora*, Sugiura (1933) noted the interval to be 11–13 days, and Ishii and Tsuda (1951), 10–14 days; for *O. quadrasi* eggs, Abbott (1946) reported a 15-day period. During the spring and summer of 1951 and 1952, the incubation period was determined for large numbers of *O. nosophora* eggs, and added information was obtained.

PROCEDURES

Female *O. nosophora* were maintained on filter paper in Petri dishes (McMullen, 1949) with decayed leaves and straw as food. Through the use of Petri dishes, necessary observations were easily made under dissecting scope. Their adequacy is indicated by the fact that adult snails have survived in them for several years with normal if not increased egg-laying.

Since a preference for soil as a laying site had been noted (Ritchie *et al.*, 1951), dried mud cubes, which could be easily removed and replaced at regular intervals, were included in the laying chambers. The eggs were laid primarily on the soil, but some were deposited on leaves and straw. They were encased in a layer of mud-like material which was characteristically molded and cemented. In some instances the egg cases were nearly submerged in the soil, if it was sufficiently wet and soft, making them inconspicuous. Consequently an exact hatching rate could not be determined, but it was estimated at 85 per cent. Subsequently it was demonstrated experimentally that 85–90 per cent of the eggs normally hatch under the laboratory conditions imposed (1954 Ann. Rept., 406th Med. Gen. Lab.).

The mud cakes and isolated eggs were transferred to incubation chambers at 24-hour intervals and, when hatching started, the young were also removed from the incubation chambers at daily intervals. Thus, there was a reciprocal error in the determination of onset and termination of incubation. Each group of eggs studied actually comprised a series of daily collections, for which the incubation interval was separately determined and the data combined.

During the summer of 1951, the incubation period was determined for 467 juveniles. In 1952 a group of eggs was collected between 10–31 March and a second between 15 May and 11 June, from which 383 and 1098 young were counted respectively.

RESULTS AND DISCUSSION

As shown by Figure 1, a few eggs hatched as early as 11–14 days after they were laid, and to this extent the observations agree with previous findings

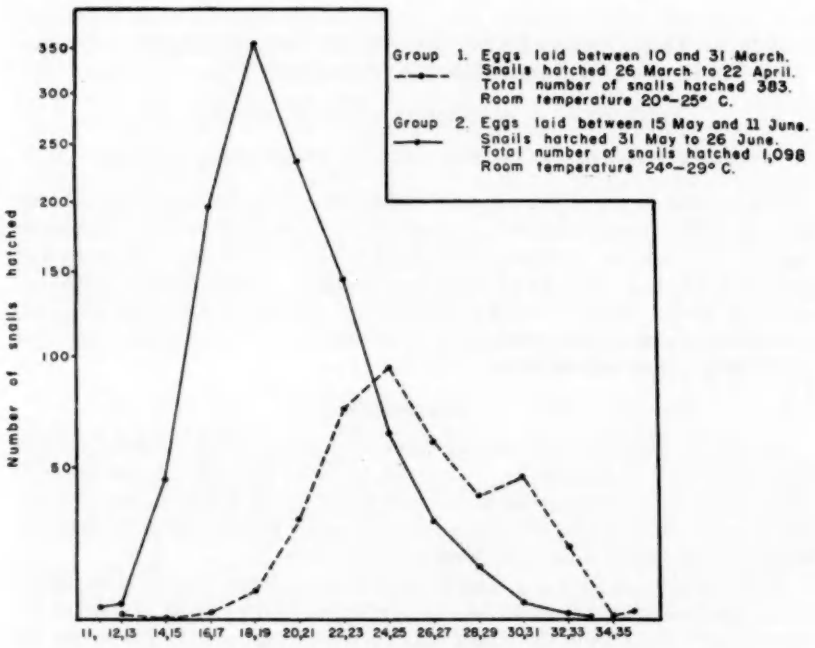
Group 1. Eggs laid between 10 and 31 March.
Snails hatched 26 March to 22 April.
Total number of snails hatched 383.
Room temperature 20°–25° C.
Group 2. Eggs laid between 15 May and 11 June.
Snails hatched 31 May to 26 June.
Total number of snails hatched 1,098
Room temperature 24°–29° C.
Number of snails hatched
350
300
250
200
150
100
50
11, 12,13 14,15 16,17 18,19 20,21 22,23 24,25 26,27 28,29 30,31 32,33 34,35
Number of days between laying and hatching.
Incubation period of Oncomelania nosophora eggs.

FIG. 1

(Sugiura, 1933; Abbott, 1946; Ishii and Tsuda, 1951). Most of the eggs, however, required a longer period of incubation, a few hatching as late as 35 days after they were laid. Distinct modal values were recognized for the two collections of eggs obtained in 1952. Maximum hatching occurred from the 24th to 25th day of incubation during March–April, when day time temperatures of 20–25°C. were recorded, while during May–June, when corresponding temperatures were 24–29°C., the peak of hatching occurred from the 18th to 19th day. This difference in the modal values was due, most likely, to the difference in temperature. For an initial experiment during the summer of 1951, the same range of time for incubation was noted, but instead of a single mode, there were two, one from the 16th to 17th day and another from the 24th to 25th day. The two experiments in 1952 invalidate the observation of the bimodal curve, with the assumption that some suboptimal condition, such as the degree of wetness, might have unknowingly and temporarily prevailed in the incubation chambers.

The marked range in the time required for incubation might, presumptively, be the result of environmental influences; however, eggs maintained within the

bounds of a Petri dish would seemingly be exposed to quite uniform conditions. This suggests the possibility of some inherent quality of the eggs. The occurrence of juveniles in the population might, then, exceed the duration of egg-laying by as much as three weeks. This staggering of hatching may constitute a safety factor against catastrophic events, as eggs and even the adults are more resistant to molluscicides than the newly hatched (1953 Ann. Rept., 406th Med. Gen. Lab.). It is also possible that eggs are more resistant to drying than the newly-hatched snail because of the manner of deposition.

SUMMARY

The incubation period of eggs of *Oncomelania nosophora* ranges from about 10 days to 35 days under seemingly uniform laboratory conditions. Maximum hatching occurred after 24–25 days at day time room temperatures of 20–25°C. in March–April, and at 18–19 days at 24–29°C. in May–June.

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STUDIES ON FLY BREEDING IN SANITARY PIT PRIVIES IN SOUTH GEORGIA

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The researches of earlier investigators have demonstrated that houseflies (*Musca domestica*) will, on occasion, feed and breed in human excrement, and may thereby become contaminated with pathogens which the flies may later transmit to man. Howard (1911), Armstrong (1914), and Watt and Lindsay (1948) have shown an apparent correlation between housefly abundance and the incidence of enteric infections. The knowledge of these epidemiological factors was the principal reason underlying the development of the so called sanitary pit privy and the advocacy of its use in unsanitated areas for the prevention of fly-borne diseases.

The plan of construction of this device is based upon the assumption that houseflies will feed and breed in the pit unless barriers are erected to exclude them. Incorporation of such fly-proofing in the plan of construction adds materially to the costs, a pertinent consideration to the low income groups which ordinarily use this privy.

Howard (1900), Graham-Smith (1914), Lorincz, *et al.* (1936), Patton (1929), Austen (1913), Dhondy (1940), Jolly (1923), Allnutt (1926), Herms (1946), Parker (1918), and West (1951) have variously discussed the breeding and feeding of houseflies in human excrement deposited on the surface of the ground, in latrines, and in unsanitary privies. All but Lorincz concurred in the opinion that a significant connection exists between *Musca* and human excrement.

The sanitary pit privy presents a quite different environment from those which were studied by the investigators cited above. The principal difference between the pit privy and surface privies and latrines is in that the former incorporates a relatively deep and dark subterranean vault which opens to the surface only through the hole in the seat, whereas, in the others, the fecal matter is deposited either on the surface of the ground or in shallow trenches or pits where it is exposed to light and air, and is freely available to insects. Observations by Paul C. Beaver (Special Report No. 91, Malaria and Hookworm Service, Georgia Department of Health, 1943) revealed the presence of *Musca* larvae in only two of 97 collections of fly larvae from sanitary pit privies. This suggested that these units are not important sources of houseflies, but unforeseen circumstances prevented a continuation of his investigations.

It appears therefore, that fly breeding in this particular type of privy has not been adequately investigated. The present study was undertaken in an attempt to determine more precisely the frequency and the conditions under which fly breeding and feeding may occur in sanitary pit privies.

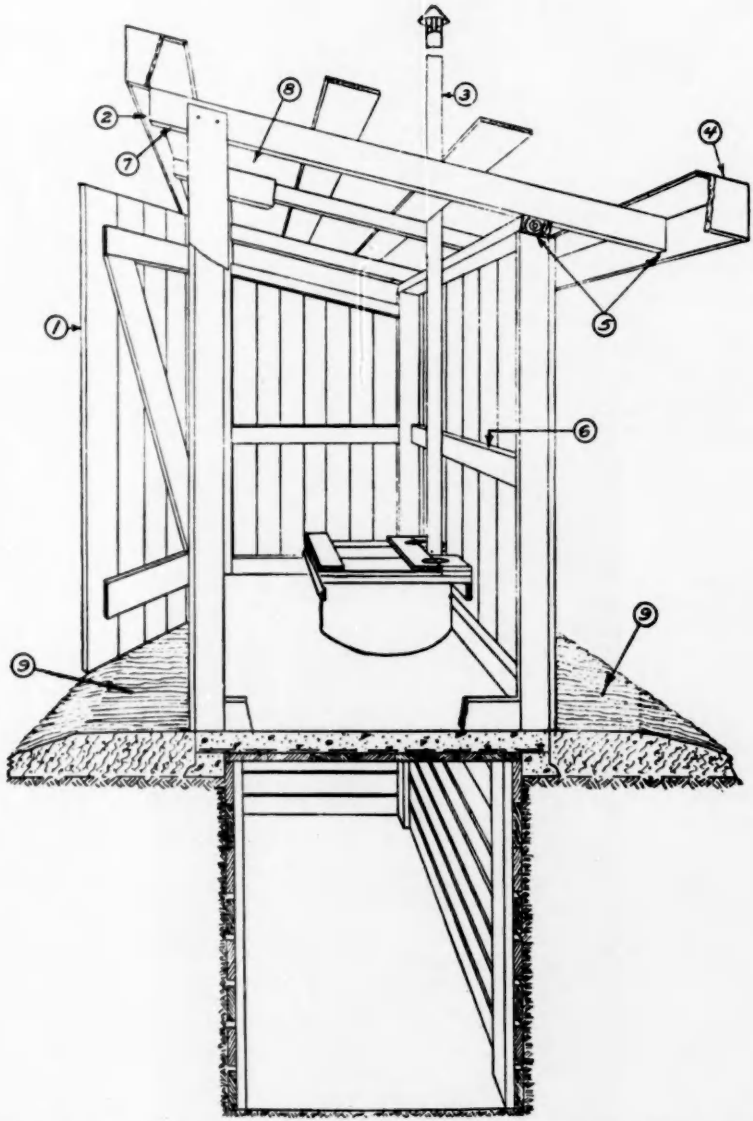

FIG. 1. Plan of sanitary pit privy. 1—door; 2 and 4—roof overhang; 3—vent; 5 and 7—rafter detail; 6—middle belt; 8—four inch opening for ventilation; 9—six inch earth mound around pit.

MATERIALS, METHODS AND RESULTS

The investigations were divided into several different phases. Materials and methods used in each, together with results and brief comments, will be presented under appropriate sub-headings.

I—Trapping insects at the riser opening to determine the frequency of their entering and leaving the pit. In this phase of the work sanitary pit privies made to the specifications described in "The Sanitary Privy" (Supplement No. 108, Public Health Reports, Gov. Printing Office, Washington, D. C.), were used as a source of insects caught in the field. Thirty of these units were selected at random: 14 were located in Dougherty County, Georgia, and 16 in Brooks County, Georgia. The total included privies which ranged all the way from new structures to some which had been in use for more than 10 years. Some were in excellent condition, and others were in various stages of disrepair. The most dilapidated of the series was one in which the building had virtually disappeared leaving little more than the seat and pit. In all, the seat cover was left open during the course of the studies to permit free entrance of insects, and, in addition to

FIG. 2. Emergence trap in position on seat riser

FIG. 3. Entrance trap

this, many had smaller or larger openings beneath the floor slab or in the riser which would give insects access to the pit. Fig. 1 shows the details of construction of the sanitary privy.

The fly traps employed to collect insects from these privies were of two different types. One, the emergence trap, was a conventional screen wire fly trap designed to fit directly over the privy seat opening in such a way that insects attempting to leave the pit below would be forced to enter the trap. Fig. 2 illustrates the design and method of use. The other trap, called an entrance trap, was also of screen wire construction, but conical in shape, and inverted in the seat opening in such a way that it would trap insects attempting to enter the pit beneath. Fig. 3 depicts this trap.

In making the collections, 20 emergence traps and 10 entrance traps were employed. Collections of insects were made twice weekly in 1950, and once each week in 1951 and 1952 over the period from June 1 to September 30. In 1950, the traps were left in position almost continuously, but, in the other two years, they were left in place for a period of 24 hours once each week. Weekly records were kept of the total number of insects caught in each privy, and species were identified microscopically.

The result of 552 collections made by means of emergence traps from 20

TABLE 1

The kinds of arthropods most commonly trapped in pit privies, the number and per cent of all collections positive for each species, and the total number of each species captured in 613 collections

Species	No. of Times Collected	Per Cent of Collections Positive	Total Number Collected
Musca domestica	56	9.1	137
Ophyra leucostoma	59	10.7	666
Hermetia illucens	135	24.5	5472
Psychoda species	72	13.0	33552
Muscina stabulans	5	1.0	54
Fannia pusio	9	1.6	114
Fannia canicularis	13	2.4	28
Dendrophaonia hilariformis	62	11.2	1384
Dendrophaonia querceti	8	1.5	23
Sarcophaga bullata	10	1.8	114
Synthesiomyia nudiseta	9	1.6	112
Meromacrus acutus	6	1.1	29
Culex quinquefasciatus	51	9.2	852
Stomoxys calcitrans	7	1.3	15
Periplaneta americana	32	5.8	127
Hymenoptera	21	3.8	764
Araneida	15	2.7	40
Orthoptera (other than roaches)	12	2.2	32
Dermaptera	8	1.5	29
Coleoptera	6	1.1	13

different privies was a total of 44,033 insects and other arthropods. One hundred and twenty seven, or 0.3 per cent of the total were *Musca domestica*. Sixty one collections from ten different privies made by means of entrance traps resulted in a total of 564 arthropods, of which 10, or 1.8 per cent, were *Musca domestica*. Forty seven different orders and species were represented in the trap collection. The insects were predominantly Diptera. Other orders included roaches, wasps and ants, beetles, earwigs, moths, bugs, termites, and innocuous spiders and centipedes. Those which occurred with any degree of frequency are shown in Table 1 which is compiled from the results of collections from both types of traps.

During the course of the study *Musca* appeared at least once in 50 per cent of the privies, but frequently only once, and always in small numbers. A total of 5 collections positive for *Musca* was the greatest number obtained from any one privy during the three year period. *Hermetia illucens* (the soldier fly) was present in virtually all of the privies, and together with *Psychoda species* (sewage flies) comprised 87.5 per cent of the total collections. *Dendrophaonia hilariformis* (a fly which closely resembles *Musca domestica*), *Ophyra leucostoma* and *Culex quinquefasciatus* were captured frequently in the traps.

American cockroaches (*Periplaneta americana*) appeared in 5.8 per cent of the trap collections, but this frequency gives little indication of their real numbers. These insects were observed in the pits of nearly all the privies, and in greater

numbers than the catches indicate. Since the fly traps were not ideally adapted to their capture, a large proportion of the roaches either escaped or evaded the traps altogether. The association of roaches with human feces, when coupled with their movements from privies to houses, may not be without epidemiological significance. *Salmonella* have been isolated from the gut of roaches captured in hospital wards and elsewhere by Mackerras (1948), and Bitter and Williams (1949). Also, Janssen and Wedberg (1952) and Jung and Shaffer (1952) have demonstrated that viable *Salmonella* persist in and may be excreted by roaches which are fed large numbers of these organisms for periods of 7 to 11 days after feeding.

II—Larvae collected from the privy pits to determine the frequency and numbers in which Musca *larvae are found in this environment.* The method used to collect larvae from the privy pit entailed the use of a long-handled dipper which was made by riveting a tin can of about one pint capacity at right angles to the end of a wooden pole of suitable length. The position of the can on the pole was determined by trial, and was found to facilitate the scraping up of material from the pit. This instrument was used at weekly intervals, to remove samples consisting of one half pint of fecal material from each privy. The larvae were removed from the material by flotation in brine solution, and preserved in 80 per cent alcohol. Ten cubic centimeters of each sample of larvae were removed from each of these collections, after thoroughly mixing the entire sample, and examined microscopically to determine species present. The total volume of larvae in each one half pint sample was then measured in a graduated cylinder and an estimation of total numbers made based upon the number of larvae observed in the 10 cubic centimeter sample.

A total of approximately 4,000,000 dipterous larvae resulted from 297 collections made from the privy pits. No larvae of *Musca domestica* were found in the collections. *Hermetia illucens* larvae made up approximately 97 per cent of the total, and, in more than 65 per cent of the privies, this was the only species present. Larvae of *Fannia*, *Ophyra*, *Psychoda* and undetermined Phoridae accounted for the remaining 3 per cent of the total.

The abscence of *Musca* larvae in the collections suggested the experiments described in the next section.

III—Planting large numbers of Musca domestica *eggs in privy pits to determine whether or not this species would complete its life cycle when introduced into this environment.* The method used for this phase of the study was merely to inoculate selected pit privies with known numbers of *Musca* eggs. One week following inoculation, and for 2 weeks thereafter, emergence traps were kept on the seat openings to trap any adult flies which emerged. *Musca* eggs used in this experiment were obtained both from our insectary cages and from the Thomasville, Georgia, laboratory of the Communicable Disease Center thru the courtesy of Dr. Dale R. Lindsay and his associates. In order to give the *Musca* every chance to develop, privies were selected in which the fecal substrate had mostly a mushy, semisolid character, with some drier areas in spots, and which appeared to offer a suitable medium for the propagation of this species.

In 1950, approximately 75,000 *Musca* eggs were introduced into each of 2 privies, and approximately 600,000 similar eggs into a third privy. The results were that no adult *Musca* emerged from the pits in which 75,000 eggs had been planted, but 347 adults emerged from the privy which had been seeded with 600,000 eggs. This figure represents a recovery of 0.06 of one per cent of the eggs introduced into the latter privy. The experiment was replicated 8 times during 1951, using privies selected as above, and introducing approximately 25,000 eggs into each unit. Seven adult *Musca* were recovered from one unit and one from another, or 0.03 of one per cent and 0.004 of one per cent, respectively, of the number of planted *Musca* eggs. The experiment was again replicated 18 times with six different privies during the summer of 1952. Approximately 15,000 *Musca* eggs were planted in each unit for each trial. No adult *Musca* were recovered from any one of the 18 trials. In order to check on their viability, some *Musca* eggs from each lot which was planted in privies were retained as controls, placed on suitable media, and reared through to adult flies. These experiments suggest that only a small number of the *Musca* eggs which might be oviposited in pit privies ever survive to maturity.

The presence of tremendous numbers of *Hermetia illucens* larvae in most of the privies suggested that the activities of these large larvae might interfere with the development of housefles in pit privies. This suggestion led to the following experiment.

IV—Rearing Musca domestica *and* Hermetia illucens *larvae together to determine whether or not the presence of numbers of* Hermetia *larvae would affect the development of* Musca *in any way.* In this study, a series of 15 one-gallon, wide-mouth glass jars was set up, each containing approximately 3 quarts of CSMA Standard Fly Larval Media (Ralston Purina Company). Approximately 500 *Musca domestica* eggs (estimated by volume) were planted on the medium in each jar. *Hermetia* larvae in various instars were obtained from privy pits and placed in each of 10 jars as follows. No. 1, 10 larvae; No. 2, 25 larvae; No. 3, 50 larvae; No. 4, 100 larvae; No. 5, 200 larvae; No. 6, 400 larvae; No. 7, 600 larvae; No. 8, 800 larvae; No. 9, 900 larvae; No. 10, 1000 larvae. Five jars in which no *Hermetia* larvae were added were kept as controls. All jars were incubated at the room temperature usual in South Georgia during the summer months. Just prior to the time for *Musca* to pupate, a layer of clean dry sand, one half to one inch in depth, was placed on top of the media to provide a dry place for pupation.

The results were that *Musca* developed and emerged in the jars which contained *Hermetia* larvae in approximately the same numbers as they did in the controls which contained no *Hermetia* larvae. From 361 to 598 adult *Musca* were recovered from each of the jars. The error inherent in measuring *Musca* eggs by volume rather than by actual count would be sufficient to explain the fact that sometimes less and sometimes more than 500 *Musca* were recovered from the rearing jars. All *Hermetia* larvae survived in apparently good condition. It was concluded that the presence of *Hermetia* larvae, even in relatively large numbers, does not inhibit development of *Musca domestica*, and the experiment was not repeated.

V—Intensity of illumination in the privy pits. Illumination in the privy pits was measured by means of a photoelectric light meter manufactured by the Photovolt Corporation, New York, N. Y. The sensitivity of this meter to light ranges from 0.01 to 500 foot candles. At each privy, measurements were made at the seat opening of the amount of light (in foot candles) reflected up from the pit. This, of course, would be the amount of light visible to an insect entering the pit through the seat opening. The measurements were made to include morning and afternoon and bright and cloudy days.

The results were that, while illumination varied greatly between the different privies in the series, it was always quite low. On bright sunshiny days, the illumination in the different pits ranged from 0.01 to 1.58 foot candles, and on cloudy days from 0 to 0.25 foot candles. The median was 0.12, and the mean was 0.31 foot candles.

DISCUSSION

The literature on Diptera is replete with conflicting reports on the habits and preferences of the housefly, which, perhaps, may be explained in part by errors in identification and the varying reactions of the species to different environments. Howard (1900) noted that several other species of flies resemble the housefly so closely that they cannot be distinguished on superficial examination even by a trained entomologist. He thought that this fact accounted for a general popular misunderstanding of the specific habits of *Musca domestica*. Austen (1926) likewise remarked that few people are capable of recognizing a housefly at sight, and that other flies are frequently mistaken for this species. It is possible that some of the previously reported associations of houseflies with human feces in pit privies have been based upon errors of this kind, or upon the failure of investigators to distinguish between pit and surface privies. Parker (1918) noted that there was a considerable difference in the species of flies trapped in shallow as opposed to deep-vaulted privies.

During the present studies, while *Musca domestica* appeared at least once in the trap collections from 50 per cent of the pit privies, this species constituted only three tenths of one per cent of the total number of insects captured in both types of traps. This figure represents an average of only one housefly per pit privy per summer for a three year period. This data, when related to the complete absence of *Musca* larvae from a total of approximately 4,000,000 fly larvae taken at random from the privy pits, suggest that sanitary pit privies are not significant sources of houseflies.

In considering possible reasons why a few *Musca* occasionally appeared in certain privies, and their consistent absence from others, an attempt was made to evaluate such factors as the age and physical condition of the privy, the character of the pit contents, the presence of rubbish, moisture, the intensity of illumination, and whether or not chemicals were added to the pit to prevent odor and insect breeding. With the exception of illumination in the pit, no really significant correlation between any of these factors and the presence of *Musca* was apparent. It was noted that in the majority of the privies which occasionally

produced *Musca* in the trap collections, the pits were filled with fecal wastes to a point within a foot or two of the surface, and in addition contained rubbish in the form of sticks, weeds, tin cans, bottles, and other items. In other words, these pits had assumed some of the characteristics of surface privies. When the pit becomes filled, the fecal substrate approaches the source of light at the seat opening, and this, of course, increases the intensity of illumination reaching the surface of the pit contents from above. However, the floor, seat riser, and building of the pit privy provide so much shade that, even in the pits which are filled to ground level, the illumination is considerably reduced. It is a matter of common knowledge, previously remarked upon by Felt (1910), Hewitt (1914), Patton (1929), and West (1951) that houseflies usually seek out well-lighted situations in which to feed and deposit their eggs. G. G. Jolly (1923) considered subdued light so unattractive to *Musca* that he thought privies should be darkened as a substitute for screening to exclude houseflies. The vault of a properly constructed pit privy is usually so dark that the aid of a flashlight is required to discern objects in the vault. It is probable that the relative darkness of the vault is the principal factor which repels houseflies from entering this environment. It should be remarked that houseflies are not only positively phototropic, but are also negatively geotropic (West, 1951), and tend to fly upwards rather than downwards. This tropism may, perhaps, play some part in discouraging *Musca* from entering the pits.

Darkness and negative geotropism, however, do not seem to be the only factors which tend to prevent the breeding of *Musca domestica* in pit privies. The fact that less than three hundredths of one per cent of a total of approximately one and one-half million *Musca* eggs which were placed in the pits ever developed into maturity suggests that some, as yet unrecognized, components of the pit environment inhibit the development of any houseflies which may invade these situations.

However, we do not intend to decry the significance of the association of *Musca* with human excrement. The studies by the investigators cited previously have demonstrated that houseflies will readily seek out this material wherever it is available in well-lighted situations, and that this association of flies with feces may result in the transmission of human disease. Our point is that the contact between houseflies and human feces appears to occur infrequently in properly constructed pit privies, but usually takes place elsewhere. There is little doubt that many other situations exist in which flies may readily avail themselves of human excrement. In a recent survey conducted in 49 Georgia counties, 67.7 per cent of all the families were found to have unsanitary surface privies, or no privy at all. Under these circumstances, houseflies have ready access to human fecal wastes, and opportunity to become contaminated with disease organisms.

To reiterate, the data resulting from the present studies indicate that houseflies neither feed nor breed in properly constructed and reasonably well maintained pit privies to any great extent. The frequency and numbers with which they were observed in these situations are small, and of doubtful significance.

Musca domestica is the only species of fly which has been seriously incriminated in the transfer of filth-borne diseases. Since *Musca* constituted more than 98 per cent of all the flies which are usually found indoors in kitchens, bakeries, and other places where food is prepared and served (Howard, 1900; Lorincz, 1936; and Haines, (1953), it is reasonable to assume that the other species of flies observed in pit privies are seldom associated with man, and are therefore of little or no public health significance.

SUMMARY AND CONCLUSIONS

The knowledge that houseflies breed and feed in human feces, and are thereby potentially dangerous vectors of disease, led to the development of the sanitary pit privy. This privy incorporates fly proof construction on the assumption that houseflies will breed and feed there unless prevented. This study undertook to ascertain the extent of such breeding and feeding. Adult flies were trapped both as they entered and emerged from the privies. More than 600 trap collections, made from a series of 30 different privies produced a total of 44,597 insects which were predominantly Diptera, but only 137, or three tenths of one per cent were *Musca domestica*. Fifty per cent of the privies produced no *Musca*. Approximately 4,000,000 larvae were collected from the pits, but no *Musca* larvae were found. It is believed that the relative darkness of the pits is the main factor which repels houseflies. The data show that the number of houseflies frequenting pit privies is small (in this study, approximately one fly per privy per summer) and of doubtful significance from the standpoint of possible disease transmission. It is concluded that fly-proofing of properly constructed pit privies is unnecessary in Georgia.

We gratefully acknowledge the assistance of Doctor H. R. Dodge in identifying Diptera.

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ERRATUM

In the article by M. T. Gillies entitled "Density of adult anopheles in the neighborhood of an East African village," and published in the November, 1955, issue of this Journal (vol. 4, no. 6, p. 1103), the following introductory paragraph was unfortunately omitted:

It has long been recognized that the coastal lowlands of tropical Africa are amongst the most malarious regions known. It was shown by Wilson (1936) and more recently by Davidson and Draper (1953) that holoendemic conditions prevail, for instance, in the northern coastal area of Tanganyika. It seemed therefore, that some knowledge of the absolute densities of the vector mosquitoes concerned in transmission in that area, *Anopheles gambiae* Giles and *Anopheles funestus* Giles, would be of value.

BOOKS RECEIVED

Diseases of the Chest, by H. Corwin Hinshaw and L. Henry Garland. 634 pp. illustrated. Philadelphia: W. B. Saunders Company, 1956. $15.00.

A Review of Work on Indian Medicinal Plants, by R. N. Chopra and I. C. Chopra. 58 pp. New Delhi: Indian Council of Medical Research, 1955. /Rs. 3/–.

Yellow Fever Vaccination, by Smithburn, Durieux, Koerber, Penna, Dick, Courtois, de Souza Manso, and Bonnel. World Health Organization, Monograph Series No. 30. 238 pp. with 37 figs. New York: Columbia University Press, 1956. $5.00.

Laboratory Manual for Microbiology, by Catherine Jones Witton. 2nd ed. 98 pp. New York: McGraw-Hill Book Company, 1956. $2.50.

World-Atlas of Epidemic Diseases (Welt-Seuchen-Atlas), by Ernst Rodenwaldt and Helmut J. Jusatz. Second part in 4 issues; 3rd issue. Hamburg: Falk-Verlag.

Manualde Micologia Médica, by Carlos da Silva Lacaz. 2nd ed. 422 pp., illustrated. São Paulo, Brazil, 1956.

Tropical Medicine for Nurses, by A. R. D. Adams and B. G. Maegraith. 314 pp., illustrated with 1 plate in color. Springfield, Illinois: Charles C. Thomas, 1955.

The Nun's Story, by Kathryn Hulme. 339 pp. Boston: Little, Brown and Company, 1956. $4.00.

Police Drugs, by Jean Rolin. 194 pp. New York: Philosophical Library, 1956. $4.75.

Précis d'Épidémiologie et Prophylazie des Grandes Endémies Tropicales, by V. Reynes. 3rd ed. Paris: Masson et Cie., 1955. 2,800 Fr.

BOOK REVIEWS

Introduction to Virology, by GILBERT DALLDORF, M.D., Director, Division of Laboratories and Research, New York State Department of Health, Albany, New York. 102 pp., illustrated. Springfield, Illinois, Charles C. Thomas. 1955. $3.50.

This book constituted a vexing problem to your reviewer because of its briefness. Briefness is an admirable trait, but it poses many problems as to the adequacy of the coverage given in any particular instance. The first eight chapters, covering 70 odd pages, deal with the following subjects: the nature of viruses and virus diseases, the pox diseases, diseases of the liver, of the respiratory tract, virus encephalitis and meningitis, poliomyelitis viruses and related agents, virus infections of the skin and brain, and finally rabies.

The large number of disease conditions covered in so few pages indicates the brevity of the presentation, yet the coverage is surprisingly good as discussion is confined to only the most important highlights and aspects. This first section of the book is, as the author points out, grossly incomplete in a number of respects, but as the book is intended only as an *introduction* to virology, this is forgivable.

The second section of about 30 pages deals with the facilities, equipment and methods of the virus laboratory and outlines some of the more common techniques employed. The aim is to give the beginner a general idea of methodology and this is, in the reviewer's opinion, rather well done.

EDWIN H. LENNETTE

Virus and Rickettsial Diseases, by S. P. BEDSON, M.D., D.Sc., F.R.C.P., F.R.S., Emeritus Professor of Bacteriology, London University; DOWNIE, A.W., D.Sc., M.D., Professor of Bacteriology, Liverpool University; F. O. MACCALLUM, B.Sc., M.D., Director Virus Laboratory Central Public Health Laboratory; C. H. STUART-HARRIS, M.D., F.R.C.P., Professor of Medicine, University of Sheffield. Second edition, 406 pp. illus. Baltimore, Williams and Wilkins Company, 1955, $6.75.

The first edition of this book appeared in 1950 and the second edition has just been issued. In the preface to the first edition, the authors pointed out that the numerous ad-

vances made in the virus field failed to come to the notice of the practicing physician because few attempts had been made to present the material in a suitable form for those whose interests are of a more general nature. Since that time, the dearth of textbooks on virology has been considerably remedied and the present book represents one of the better texts.

The authors have attempted to present "a coherent, readable account rather than an exhaustive treatise." This has, on the whole, been rather well achieved despite the brevity of some of the chapters. For example, the Coxsackie viruses are covered in five pages and the arthopod-borne virus encephalitides, in 24 pages. However, much new material has been incorporated. The chapter on poliomyelitis has been rewritten, and information on Murray Valley encephalitis has been included in the section on arthropod-borne viral encephalitides. The book is as up-to-date as was possible at the time of printing, including, for example, mention of the new respiratory disease viruses recovered by tissue culture methods, and describing, in the chapter on poliomyelitis, agents which have come to be called "orphan" viruses and recently included in a group referred to at the moment as "ECHO" viruses.

The book gives a very good, if short, discussion of viral and rickettsial diseases of man and can be recommended as a teaching text in this field.

EDWIN H. LENNETTE

A Textbook of Medicine edited by RUSSELL L. CECIL, M.D., Sc.D., Professor of Clinical Medicine Emeritus, Cornell University, New York and ROBERT F. LOEB, M.D., Sc.D., D. Hon. Causa, LL.D., Bard Professor of Medicine, Columbia University, New York. Associate Editors: ALEXANDER B. GUTMAN, WALSH MCDERMOTT AND HAROLD G. WOLFF. Ninth Edition: 1,786 pp., illustrated, with many in color. Philadelphia, London: W. B. Saunders Company, 1955, $15.00.

The ninth edition of this standard Textbook of Medicine can be highly recommended. For those who do not have one of the later editions and who wish to remain familiar with advances in the field of Internal Medicine, this latest volume is essential. In recent years so much new information has become available that even such a complete text as this can do no more than "hit the high spots," especially where the less common clinical conditions are concerned. This edition, however, maintains a good balance in coverage of material, yet gives adequate attention to all but the most esoteric conditions. The key references at the end of each article will prove helpful to the reader. The reviewer was pleased to note that almost every list contained at least one recent reference article, which indicates that the authors must have been revising their copy until the moment the book went to press.

In general, the format of previous editions is followed, with double column pages and readable print, but with increased use of diagrams. The illustrations are quite adequate, although this reviewer hopes that more color plates can be used in subsequent editions. The reproductions of x-rays are good, but the lack of indicators pointing to the involved areas might handicap the novice. The reviewer wishes that the reproductions of electrocardiographic tracings could be improved, so that both the figures and the explanatory material could be understood with greater ease.

This edition contains 39 new articles and 58 complete revisions of subjects previously covered. As one would expect in a text representing the work of 172 contributors, the presentation of material is uneven, but the editors have done a magnificent job so that the continuity is quite good. In general, there is adequate coverage of the necessary physiologic and biochemical background material.

The section on infectious diseases, which should be of great interest to readers of this journal, seems well done to one who is not an expert in the field. The problem of keeping up to date with the latest therapeutic discoveries is handled as well as possible in a textbook that must meet a publication deadline. The sections on protozoan and metazoan infections seem excellent for a textbook of general medicine.

The section on collagen diseases, which are of so much current interest, provides a good background in this field, where a great deal of our knowledge is of recent origin.

The sections on diseases due to physical and chemical agents is most helpful to the physicians who must be aware of the problems that air travel might pose for his patients, who must treat cases of accidental and suicidal poisoning, who needs to know something about industrial chemical hazards and who must take his place in civil defense plans. The articles dealing with alcoholism and narcotic addiction are excellent. Although poisoning with cholinesterase inhibitors is discussed in the section on nerve gases, problems raised by the newer insecticides might be covered in greater detail.

The section on diseases of metabolism is of high caliber, and includes excellent treatises on gout and diabetes mellitus and adequate coverage of the rarer conditions. The reviewer wonders whether it is advisable to discuss alkalosis, acidosis, dehydration and fluid balance under separate headings, rather than to integrate them in a single article.

The section on diseases of the ductless glands begins with a stimulating introduction and surveys the field in a satisfactory manner. The reader whose knowledge of this subject is somewhat lacking will find this section most helpful.

Diseases of the digestive system are well covered, with a good deal of space devoted to diseases of the liver.

The article on pulmonary function in health and disease in the section on diseases of the respiratory system is extremely well done. It will be of particular value to those who feel they have lagged behind in their knowledge of the newer methods of measuring and evaluating pulmonary function.

A competent discussion of diseases of the kidneys is followed by a short review of renal physiology and tests of renal function. This section includes articles on hemoglobinuria, myohemoglobinuria, and hemolytic transfusion reactions.

The section on diseases of the blood is generally informative; the articles on anemias and hemorrhagic diseases are particularly helpful.

The sections on diseases of the cardiovascular system and diseases of the peripheral vessels offer a well-rounded survey of this broad field of medicine. The article on the pathologic physiology of generalized circulatory failure and that on general considerations of diseases of the peripheral vessels will be particularly useful to the average reader. Thorough discussions of congenital heart disease and mitral stenosis reflect current interest in the possible surgical treatment of these conditions.

The complete coverage of the arthritidies under diseases of the locomotor system will be helpful to physicians who are interested in the treatment of such conditions.

The section of diseases of the nervous system includes a discussion of important symptoms and signs of nervous system diseases, then specific articles on diseases of the brain, spinal cord, nerve roots, etc. A general consideration of the neuroses and psychoses is included, as well as a brief orientation article on psychosomatic medicine. The reviewer believes that many physicians will find much of value in this section. A more extensive use of diagrams might be recommended for future editions.

Sections on allergy, on deficiency diseases, the spleen, and on the reticuloendothelial system round out the general coverage.

This textbook can serve the interest and needs of students and physicians of varied levels of training and experience. It is almost a superhuman task to give a well-rounded survey of the field of general medicine in one volume, but the editors and contributors have been successful in this most difficult assignment.

ROBERT H. CREDE

Origins of Resistance to Toxic Agents—Proceedings of the Symposium held in Washington, D. C., March 25-27, 1954. Ed., M. G. SEVAG, Department of Microbiology, School of Medicine, University of Pennsylvania; ROGER D. REID, Head, Microbiology Branch, Office of Naval Research; and ORR E. REYNOLDS, Director, Biological Sciences Division, Office of Naval Research, Department of the Navy. With 28 contributors. 471 pp. New York: Academic Press, Inc., 1955. Price $12.00.

This volume has both the merits and defects that characterize most symposia publica-

tions in that the wealth of viewpoints expressed stimulates the reader, but leaves a bewildering number of unanswered questions and unresolved contradictions. The various sections are addressed, of course, to specialists and hence, will be most useful to researchers in the various fields and to advanced students. Five sessions are reported, devoted respectively to resistance to microbicides; resistance to herbicides and insecticides; tolerance and addiction to drugs and alcoholism; resistance factors, infectious agents and cancer cells; physiological, chemical and genetic viewpoints. The reports of the question and answer period held at the end of each session serve to bring out viewpoints of participants in the symposium, other than the formal speakers.

Despite the diversity of organisms concerned, certain ideas seem to be common regarding the phenomena of resistance. Thus, the feeling was prevalent at one time that the future of chemotherapy of bacteria was "in a precarious balance between the discovery of new antibiotics and the exploitation by microbial pathogens of their latent capacities to develop resistance." Substituting insect pests for microbial pathogens and insecticides for antibiotics in this sentence gives an accurate statement of the feeling of many entomologists when resistance to DDT and a number of other insecticides was first proven beyond argument. Happily, the discouragement in both camps has been replaced by a feeling that resistance provides an interesting challenge which can be met successfully. A second widely held concept concerns the various possible factors involved in the development of resistance, such as reduction in permeability of cell walls, increase in detoxifying mechanisms and development of alternate metabolic pathways.

On the other hand, several speakers expressed the belief that resistance can arise in microorganisms without selection, e.g., Schnitzer says, "methods that reduce the incidence of selection are generally more suitable for producing drug resistance, at least in trypanosomes." This is in contradiction to the statement of Chadwick (concurred in by practically all who have worked on resistance in insects), "Resistance is the consequence of selection pressure exerted by the presence of the toxicant in the environment."

It is impossible to mention many masterly presentations of individual subjects but to this reader the two chapters in the last section—Biochemical genetics in relation to the action of drugs and inhibitors, by H. K. Mitchell and Protein molecule resistance to microbicides, mutations, and related problems by M. G. Sevag are outstanding.

Altogether, this is a very meritorious volume which should be close at hand to every one interested in any aspect of resistance to toxic action.

W. M. HOSKINS

Nutrition in India, by Dr. V. N. PATWARDHAN. 345 pp. Bombay: The Indian Journal of Medical Sciences, 1952. Rs 15-8.

This book is, as the author states in the preface, "a review of work done in India in nutrition and allied subjects during the last forty years or more." As such it deals with Indian foods—their production, preparation, and nutritive value; the nutritional status of Indian children as shown by nutrition surveys; basal metabolism studies of Indian adults; descriptions of deficiency and dietary diseases found in the country; and finally, a brief history of nutrition in public health practice and the author's views on the question of population increase in relation to food supplies.

Although the presentation is not highly technical, it is obviously intended for readers with a knowledge of nutrition. The book should be useful in orienting health workers to the food and nutrition situation in India. Research workers may find in the book references to work either not previously published or published only in reports not readily available. The informed reader will question some of the findings reported, such as the increased nitrogen retention found in subjects consuming vegetable protein as compared to that in subjects on animal protein diets, and the possible relationship of polluted water to goiter incidence. Nevertheless, the book well deserves attention for its presentation of material not readily available elsewhere.

RUTH HUENEMANN

Dried BCG Vaccine, by YOJI OBAYASHI, M.D., Japan Anti-Tuberculosis Association, Tokyo, Japan. 220 pp. WHO Monograph Series No. 28. Columbia Univ. Press, International Documents Service, 2960 Broadway, New York 27, N. Y. 1955. $5.00 (cloth).

The sensitivity of liquid BCG vaccine to light and temperature complicates its use in mass vaccination campaigns against tuberculosis, especially in tropical regions. Freeze-drying applied to BCG gives a more stable product but the vaccine loses some of its viability before becoming stabilized. It still retains high antigenic potency for at least a year in the refrigerator but after two years loses a certain amount. There are many compensating advantages over a liquid vaccine, but dried BCG as prepared at present does not fulfill all conditions for practical application on a large scale since it must be kept at below 5°C. This is the chief problem now under investigation.

L. W. HACKETT

Chemotherapy of Malaria, by G. COVELL, G. R. COATNEY, J. W. FIELD AND J. SINGH. 121 pp., (French edition in preparation). Geneva, World Health Organization: Monograph Series, No. 27. 1955. $3.25 (cloth) 17/6, Sw. fr. 10.–

Until the end of World War I, quinine and other cinchona alkaloids were the only specific drugs available for the treatment of malaria, but in recent years the development of synthetic antimalarials has proceeded so rapidly and successfully that "there are few infections more responsive to well-directed therapy than human malaria, and fewer in which therapy can be applied with results so satisfying." The authors, appointed to the task by the World Health Organization, present an authoritative assessment of the properties and potentialities of the newer drugs which will be particularly useful to the medical profession. This small book is very comprehensive, giving within the compass of about 100 pages a historical review, definitions of terms, a practical description of the compounds in common use, a discussion of drug resistance and a guide to the clinical use of antimalarial drugs. The book contains a selected bibliography of about 200 titles, and a good index.

L. W. HACKETT

An Attempt to Control Leprosy by B.C.G. Vaccine in the Loyalty Islands, by Médecin-Capitaine des Troupes Coloniales LACOUR, Directeur de L'Institut Pasteur de Nouméa. 45 pp., with maps and charts. Paper cover. Mimeographed. Sydney, Australia, Box 5254 G.P.O. The South Pacific Commission. 1955.

An attempt to control leprosy through the use of B.C.G. vaccine was started in 1954 in the Loyalty Islands, chosen because endemic leprosy has been studied in New Caledonia and dependencies for 50 years and annual case-finding records are available. This report summarizes the working procedures, which comprise Mantoux and Mitsuda testing, and B.C.G. vaccination by skin scarification. Early results, presented in graph form, show tuberculin and lepromin indices and the nature of the reactions and various correlations. It has not been possible to formulate any conclusions, which must naturally await a long period of observation.

L. W. HACKETT

"Map: World Distribution of Spirochaetal Diseases. 3. Leptospiroses, Plate 17, Atlas of Diseases. New York: American Geographical Society. 1955. $1.25 folded, $1.50 flat.

The Briesemeister Elliptical Equal-Area Projection adopted may be esthetically satisfying to cartographers but is markedly inferior to the distorted but useful Mercator projection in avoiding meaningless clutter of superimposed symbols. The references listed on the reverse of the map are incomplete and nondiscriminating, e.g., omission of "Leptospirosen" by Gsell and Wiesmann (1952) and "The Laboratory Diagnosis of Leptospirosis" by Wolff (1954), and failure to include primary references in the United States. The section entitled Epidemiology is a hodge-podge of assumptions, mis-statements and mistakes. It is believed that omission of this plate from the Atlas would materially enhance its value.

WILLIAM S. GOCHENOUR, JR.

Animal Agents and Vectors of Human Disease

By ERNEST CARROLL FAUST, M.A., Ph.D.

The William Vincent Professor of Tropical Diseases and Hygiene and Head, Division of Parasitology, Department of Tropical Medicine and Public Health, The Tulane University of Louisiana, New Orleans

PARASITISM constitutes one of the major phenomena of the living world, and therefore one of its most interesting and challenging subjects. A highly important phase of parasitology is the broad area involving *animal agents and vectors, and their relation to disease.* Dr. Faust, internationally known for his work in this field, presents a full discussion of those parasites which produce human disease. The etiology, epidemiology, pathogenesis, symptomatology, diagnosis, treatment, and control of this group of infections are considered in detail.

The presentation is intentionally not exhaustive, yet the volume is much more than an outline syllabus. In extensive, helpful tables in the introductory chapters, Dr. Faust tells where each parasite under discussion is found, its usual infective stage, portal of entry, primary and secondary sites, usual reservoir hosts (monkeys, dogs, rats, etc.), and other obligate hosts and vectors. Pathologic effects and clinical manifestations produced by animal agents of disease, how to diagnose them, the most satisfactory therapeutic procedures and methods of control, etc., are presented in a similar manner. Subsequent chapters give detailed information on each agent or vector as it is considered individually.

Morphologic features of the organisms involved and the clinical aspects of the diseases which they produce, are elucidated by numerous helpful illustrations, many of which were made for this book. Ample references conclude each chapter. This important and timely work is designed for use by zoologists, epidemiologists, pathologists, practicing physicians, laboratory diagnosticians and public health workers.

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